The MAPK p38δ Regulate Glucose Homeostasis

by Suppressing Insulin Secretion from Pancreatic β Cells

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1. Abstract

Decreased peripheral insulin sensitivity in combination with reduced function and mass of insulin producing β cells are the main pathogenic factors of type-2 diabetes mellitus[1-3]. Recent studies suggest that increased activity of the mitogen-activated protein kinase (MAPK) c-jun N-terminal kinase (JNK) in response to inflammatory and metabolic stresses represents a central signal transduction event promoting peripheral insulin resistance[4, 5]. JNK activation also attenuates insulin production and secretion and increases apoptosis of islet cells[6-8], several factors, which contribute to impaired glucose homeostasis in diabetic patients. The closely related MAPK p38 family consists of four members p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12) and p38δ (MAPK13)[9]. However, the specific role of each isoform remains poorly understood. Here, we demonstrate that mice lacking p38δ (MAPK13) show improved glucose tolerance due to enhanced insulin secretion caused by increased insulin granule exocytosis in pancreatic β cells. Deletion of p38δ results in enhanced formation of vesicles at the Golgi apparatus. Conversely, expression of constitutively active p38δ promotes generation of tubular Golgi protrusions. Enhanced vesicle formation is associated with pronounced activation of protein kinase D (PKD), a critical step at the trans-Golgi network (TGN) required for budding of secretory vesicles. Inhibition of PKD restores the Golgi integrity and is sufficient to reconstitute insulin secretion of p38δ-deficient islets and glucose tolerance in p38δ null mice. Hence, p38δ links TGN function to insulin secretion and might represent a novel therapeutic target in type-2 diabetes.

2. Synopsis

Das Auftreten von Diabetes Mellitus II ist in den vergangenen zwei Jahrzehnten dramatisch angestiegen.

In meiner Arbeit untersuchte ich die molekularen Mechanismen, die zur Entstehung von Diabetes II führen. Ich identifizierte ein Protein (p38δ) als Inhibitor der Insulinsekretion. p38δ ist damit ein mögliches Ziel für die Behandlung von Diabetes II.

3. Abbreviations

ATP	adenosine tri-phosphate
CNS	central nervous system
CVD	cardiovascular disease
DAG	diacylglycerol
ER-stress	endoplasmic reticulum stress
FFAs	free fatty acids
ERK	extracellular regulated kinase
GLP-1	glucagon like peptide 1
GPCR	G protein coupled receptor
GSK3	Glycogen-synthase kinase-3
GTT	glucose tolerance test
IGF-1	insulin-like growth factor 1
INS1	rat insulinoma cell line
IR	insulin receptor
IRS	insulin receptor substrate
ITT	insulin tolerance test
JNK	c-jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MIN6	murine insulinoma cell line
MODY	maturity onset diabetes of the young
NFκB	nuclear factor kappa B
PKA	protein kinase A

PKD	protein kinase D
TGN	trans-Golgi network
TNFα	tumor necrosis factor α
TLR	Toll-like receptor

4. Introduction

4.1. Diabetes mellitus – general aspects

Diabetes mellitus represents one of the most common uncommunicable diseases affecting almost 6% of the world's population. According to the International Diabetes Federation, the number of diabetic patients is predicted to reach 300 millions in 2025[10].

Diabetes is defined as a state in which carbohydrate and lipid metabolism is inappropriately regulated by insulin. This results in elevated fasting and postprandial glucose and eventually leads to multiple clinical complications[11]. There are two main forms of diabetes mellitus. Type-1 diabetes mellitus is well recognized as a condition of absolute insulin deficiency due to extensive autoimmune destruction of pancreatic β cells[12]. Type-2 diabetes mellitus is characterized by several metabolic defects, among which β cell secretory dysfunction and peripheral insulin resistance are considered as hallmarks of the disease[13]. Previously, type-2 diabetes was regarded as a distinct disease entity, but in reality, it is often a manifestation of a much broader underlying disorder called the metabolic syndrome – a cluster of cardiovascular disease (CVD) risk factors that, in addition to glucose intolerance, includes hyperinsulinaemia, dyslipidaemia, atherosclerosis, hypertension, visceral obesity, hypercoagulability and microalbuminuria[14]. The correlation of the occurrence of these conditions suggests a high interconnectivity of these traits and implies interdependent cellular and molecular mechanisms.

Elevated plasma levels of glucose in type-2 diabetic individuals result from disruption of equilibrium between deposition of glucose in peripheral insulin-sensitive organs, mainly skeletal muscles and liver, absorption of carbohydrates and other nutrients in the gastrointestinal tract as well as output of glucose from the liver[11]. This equilibrium is regulated

mainly by two hormones: insulin, secreted from the pancreatic β cells to circulation during the state of high glucose plasma levels which promotes glucose deposition; in contrast, glucagon, secreted by pancreatic α cells during periods of fasting, promoting gluconeogenesis in the liver[15].

As mentioned above, type-2 diabetes mellitus is mostly associated with visceral obesity. Obesity is caused by a dysbalance between energy intake and energy expenditure and results in energy deposition in form of fat in adipocytes. The mechanisms controlling energy intake involve both the control of nutrient uptake in the gut and the regulation of appetite, the latter of which is controlled centrally and involves a hormonal communication between the brain and peripheral tissues[16]. Energy expenditure occurs through physical activity (voluntary movements), basal metabolism (myriad of biochemical processes necessary to maintain life) and mainly in rodents through adaptive thermogenesis (energy dissipated in the form of heat in response to environmental changes)[17].

A causal contribution of obesity to type-2 diabetes mellitus is reflected by the fact that normalization of the body weight restores several metabolic disturbances. Body weight reduction therefore constitutes a main therapeutic goal in diabetic patients. As a consequence mechanisms controlling whole body energy homeostasis and adipose growth, build the focus of many research laboratories. Adipose accretion and the metabolic overload of adipocytes activates a number of stress-induced pathways to ultimately increase secretion of multiple insulin resistance factors including inflammatory mediators (TNFα, IL-6)[18-20], free fatty acids (FFAs)[15] and insulin desensitizing adiponectins such as for example resistin[21]. In the same time, adipose-derived insulin sensitizing factors such as adiponectin tend to be secreted in reduced amounts[22]. Several studies provided evidence that these factors are key in promoting or protecting against insulin resistance, respectively. Decreased peripheral insulin sensitivity initially is compensated by increased insulin production and secretion from pancreatic β cells. These adaptive mechanisms are however limited since inadequate expansion of those cells or an

inappropriate response to high glucose levels result in β cell failure at later stages. Altogether, decreased mass and function of β cells in combination with insulin resistance are key factors to promote severe glucose intolerance leading to elevated fasting glucose plasma levels characterizing type-2 diabetes mellitus[15].

4.2. Mechanisms of insulin resistance

Peripheral insulin resistance is defined as a state when insulin action is blunted[15]. Insulin is the dominant hormone regulating plasma glucose level. Generally, insulin promotes glucose uptake mainly in skeletal muscle, liver and adipose tissue. Moreover, it promotes storage and synthesis of lipids, proteins and carbohydrates and inhibits their breakdown[23]. In the different cell types and organs, insulin regulates specific processes. In liver, it promotes glycogen synthesis and inhibits gluconeogenesis. In adipose tissue, it has been demonstrated to suppress lypolysis and the release of free fatty acids to the circulation[24]. In the central nervous system, insulin receptors have been shown to be widely expressed. Several actions of insulin in the brain have been reported as well. Insulin suppresses appetite and counteracts CNS-mediated hepatic glucose production[16].

At the molecular level, binding of insulin to its receptor evokes a cascade of several molecular events including translocation of the glucose transporter 4 (Glut-4) from cell interior to the plasma membrane to allow glucose uptake, phosphorylation of glycogen-synthase kinase-3 (GSK3) to promote glycogen formation[25], inhibitory phosphorylation of FOXO-1 and/or FOXA2 to inhibit gluconeogenesis and/or β oxidation of FFA[26-29], activation of mammalian target of rapamycin (mTOR) to promote protein synthesis and various molecular events promoting lipogenesis and preventing lipid break-down[13].

The first event leading to cellular effects upon insulin binding to the insulin receptor (IR) is its conformational change and its autophosphorylation on multiple tyrosine residues including tyrosine 960 generating a recognition motive for the phosphotyrosine-binding domain (PTB domain) of insulin receptor substrates (IRSs)[23, 30]. The function of the IR in regulation of glucose homeostasis was studied extensively. Mice with a liver-specific deletion of the insulin receptor (LIRKO mice) exhibit dramatic insulin resistance and glucose intolerance leading to

progressive hepatic dysfunction[31]. Very recently, LIRKO mice have been shown to produce dyslipidemia and susceptibility to atherosclerosis[32]. Surprisingly, deletion of the insulin receptor specifically in skeletal muscles (MIRKO mice) did not alter blood glucose levels[33]. In contrast, deletion of the insulin receptor in adipose tissue (FIRKO mice) resulted in a reduced fat pad mass, reduced triglyceride body content, decreased body mass and protection against obesity and glucose intolerance[34]. Furthermore, FIRKO mice show prolonged longevity[35]. Taken together, these data suggest a complex tissue-dependent function of insulin receptor signaling.

At least 12 substrates for the insulin receptor have been identified so far. The most characterized are IRS-1 and IRS-2[14]. Deletion of IRS-1 in mice results in growth defects, severe insulin resistance and glucose intolerance[36]. Similarly, targeted disruption of IRS-2 results in development of diabetes[37].

Upon phosphorylation on tyrosine residues, IRSs can interact with the regulatory subunits p85, p55 and p50 of the phosphoinosytol 3-kinase (PI3K). These regulatory subunits in turn bind to the catalytic subunits (p110) of PI3K which in turn converts phosphatidylinositol 4,5-diphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3)[23]. Activation of PI3-kinase seems to be crucial for the translocation of Glut-4 to the plasma membrane since its blockage with specific inhibitor wortmannin markedly attenuates this process[38]. Mice with deletion of the p85α regulatory subunit of PI3K show increased glucose tolerance and hypoglycemia since ablation of p85α leads to an overall increased PI3K activity[39]. Conversely, deletion of the catalytic subunit results in insulin resistance[40].

PIP3 generation results in the activation of multiple downstream targets including phosphoinositide-dependent kinase 1 and 2 (PDK1 and 2) and subsequent activation of AKT isoforms 1 to 3 (also known as protein kinase B, PKB) and protein kinase C (PKC) ζ. Interestingly, PDK2 has been recently discovered to be the rapamycin-insensitive mTOR-Rictor complex[41]. Activated AKT in turn phosphorylates several targets including the AKT substrate

of 160kDA (AS160), the latter of which mediates GLUT4 translocation and regulates GSK3β and FOXO1 to control glucose homeostasis[13, 23, 30].

In general, impaired insulin signaling leads to insulin resistance characterized by an impaired response to insulin in peripheral organs such as in liver, skeletal muscle and adipose tissue. Several molecular mechanisms accounting for attenuation of insulin action have been proposed. In early nineties, studies from Bruce Spiegelman's and Gökhan Hotamisligil's laboratories showed that tumor necrosis factor α (TNFα) is secreted in higher amounts from the adipose tissue of obese animals. Moreover, these studies showed that neutralization of TNFα increased insulin sensitivity and improved glucose tolerance[42]. Since this initial study implicating inflammation in obesity-related type-2 diabetes, multiple other lines of evidence have arisen to support this concept. First of all, increased TNFα abundance in the tissues derived from obese humans was shown[43]. Moreover, other inflammatory mediators such as interleukin-6 (IL-6) and monocyte chemoatractant protein-1 (MCP-1) have been demonstrated to mediate insulin resistance in obese mice[20].

As mentioned above, adipocytes secrete adipokines such as leptin, visfatin, resistin and adiponectin, which regulate different aspects of metabolism and expression of multiple inflammatory mediators[44]. Adiponectin has been shown to generally promote insulin sensitivity and mediate anti-inflammatory effects[44]. Leptin is a master regulator of appetite. Mice deficient either in leptin (*ob/ob* mice) or leptin receptor (*db/db* mice) displays markedly increased food intake and severe obesity. In fact, mice carrying those mutations are widely used as animal models for studying the development of obesity and associated disorders[45]. Moreover, leptin is a potent regulator of proinflammatory mediators, promoting TNFα, IL-6 and IL-12 expression as well as neutrophil chemotaxis and T effector cells differentiation[45]. The recently discovered adipokine resistin has been shown to promote expression of TNFα and IL-6 and can be classified as insulin desensitizing factor[46]. In contrast, visfatin, an other newly identified

adipokine, promotes insulin sensitivity by direct interaction with the insulin receptor[44]. Deregulation of the secretion of adipokines, especially a decrease in secretion of adipokines promoting insulin sensitivity and an increase in secretion of insulin desensitizing factors during obesity, contributes to development of insulin resistance[44-46].

Several recent studies suggest that obesity is associated with an increase of pro-inflammatory macrophages migrating to the adipose tissue, which in concert with metabolically-overloaded adipocytes contribute to a low-grade inflammation[47-49].

As a consequence of reduced insulin signaling, glucose uptake is decreased and insulin-mediated suppression of lipolysis is attenuated. Additionally, dysfunctional adipocytes exhibit a reduced rate of triacylglycerol synthesis and accelerated lipolysis[50], both of which are resulting in increased cellular efflux of free fatty acids (FFA). Increased lipotoxic FFA and systemic inflammation in turn may affect insulin sensitivity and lipid metabolism in liver and skeletal muscle leading to systemic insulin resistance and dyslipidemia and finally also might contribute to β cell dysfunction (see next chapter), all of which are pivotal to the development of type-2 diabetes and associated disorders including atherosclerosis[51 52].

Taken together, several studies confirmed that a low-grade chronic and systemic inflammatory state in the adipose tissue, dysbalanced adipokine secretion, as well as enhanced FFA release from adipocytes are key features in obese subjects mechanistically connecting adipose accretion with insulin resistance, dyslipidemia and finally impaired β cell function.

However, the question remains how these factors suppress insulin action at the molecular level. For quite a while, it is fairly established that infectious agents and other environmental and cellular stresses trigger a number of metabolic responses. In fact, an acute infection leads to acute insulin resistance to elevate blood glucose levels, which is necessary to provide sufficient energy to immune cells, which are mainly dependent on glycolysis as a source to generate ATP[53, 54]. Furthermore, acute infections lead to a plethora of physiologic alterations in lipid metabolism[55-57]. However, if inflammation and other stresses are sustained,

as for example in obesity, these responses can become detrimental and lead to life-threatening diseases such as cancer, diabetes and atherosclerosis. Among others, two main canonical pathways mediate the cellular responses to inflammation and other stresses, the Mitogen-activated protein kinase (MAPK) and the nuclear factor kappa B (NFκB) pathway. These pathways appear to be chronically and highly activated in metabolically overloaded tissues[4, 58]. Moreover, several studies in knockout mice provide evidence that these signaling cascades are required to promote obesity-related insulin resistance. It was demonstrated that the MAPK JNK1, but not JNK2, promotes obesity and insulin resistance[4]. However, very recent data from the same research group provide evidence that also JNK2 is involved in metabolic regulation, but its function is compensated by JNK1[59]. At the molecular level, JNK directly suppresses insulin signaling through a phosphorylation-induced inactivation of the insulin receptor substrate 1 (IRS-1) at Ser307[60]. JNK1 has been also shown to trigger inflammation in adipose macrophages to promote obesity-related insulin resistance[5]. Stress-induced JNK activation also occurs in macrophages residing in the vascular wall, which enhances their capacity to take up modified lipoproteins and thus promotes foam cell formation, a key process in atherogenesis, the latter of which is frequently associated with obesity and type-2 diabetes [61].

The nuclear factor kappa B (NFκB) signaling cascade represents another canonical pathway activated by stress and inflammatory agents[62]. Activation of most forms of NFκB, especially the most common form - the p50–RelA dimer - depends on phosphorylation-induced ubiquitination of the IκB proteins, the latter keeping NFκB in its inactive state. This sequential modification depends on two protein complexes: the IκB kinase (IKK) complex and the $E3^{IκB}$ ubiquitin ligase complex[63]. Once poly-ubiquitinated, the IκBs undergo rapid degradation through the 26S proteasome and the liberated NFκB dimers translocate to the nucleus, where they participate in transcriptional activation of specific target genes. IKK is composed of at least three subunits: the catalytic subunits IKKα and IKKβ, and the regulatory subunit, IKKγ.

The IKKα and IKKβ subunits preferentially form heterodimers, and both can directly phosphorylate the critical S32 and S36 residues of IκB. IKKγ serves a structural and regulatory purpose and is thought to mediate interactions with upstream activators of IKK in response to cellular activation signals[64].

Importantly, IKKβ seems to promote obesity-related insulin resistance. In fact, heterozygous deletion of IKKβ protected against the development of insulin resistance during high fat feeding and in obese leptin deficient mice[58]. Consistently, it has been demonstrated that mice with macrophage-specific deletion of IKKβ retained insulin sensitivity in peripheral organs and were protected against insulin resistance in response to high fat diet and genetically-induced obesity. Moreover, liver-specific expression of a constitutive active form of IKKβ led to a subacute chronic inflammatory response in this organ and to the development of type-2 diabetes in mice[58]. Deletion of IKKβ in insulin-responsive tissues had further illustrated its role in metabolism. Although hepatocyte-specfic deletion of IKKβ in mice does not affect liver insulin responsiveness, it results in insulin resistance in muscles and fat[65]. Muscle-specific inactivation of IKKβ does not affect insulin sensitivity in mice[66]. Deletion of other component of the NFκB signaling IKKγ specifically in liver results in protection from obesity-induced development of insulin resistance[67]. Taken together, these results suggest that activation of NFκB promotes insulin resistance although direct interaction with the insulin signaling cascade was not reported. It seems that activation of NFκB promotes inflammatory cytokine expression and indirectly attenuates insulin signaling.

These stress-induced kinases also mediate innate immune responses via signaling cascades induced by the Toll-like receptors (TLRs). Pathogen-associated molecular pattern (PAMP) recognition by Toll-like receptors (TLRs) constitutes a central mechanism for initiation of innate immune responses and the induction of pro-inflammatory cytokines and type I interferons during infection[68]. Intriguingly, it has been shown that FFA bind the toll-like

receptor TRL4[69, 70]. As FFA are frequently elevetated in sera of obese subjects, binding of FFA on TLR4 might promote obesity-related inflammation[71]. Indeed, deletion of TLR4 resulted in protection against obesity-induced insulin resistance[56, 71, 72]. Therefore, these data might explain how altered lipid metabolism is converted into a inflammatory stress program in adipocytes. Another upstream mechanism activating JNK and IKK in metabolically-overloaded tissues is the induction of endoplasmic reticulum stress (ER-stress)[54]. Mice with heterozygous deletion of the X-box binding protein 1 (XBP-1), a transcription factor which enhances transcription of ER chaperones and thus attenuates ER-stress, develop insulin resistance[73]. In contrast, in vivo delivery of chemical chaperones attenuates ER-stress in obese animals, thereby improving insulin sensitivity[74].

In conclusion, the following simplified and adipocentric model of obesity-related insulin resistance can be proposed: Enhanced nutrient intake and reduced energy expenditure leads to storage of fat in adipocytes. Metabolically-overloaded adipocytes aberrantly induce inflammatory pathways, secrete higher amounts of insulin-desentizing adipokines (i.e. resistin), decreased amounts of insulin-sensitizing adipokines (i.e. adiponection and visfatin) and release markedly more FFA mainly by increasing lipolysis. These factors might impair the metabolic function of other organs such as liver and skeletal muscle leading to systemic insulin resistance (Fig. 1).

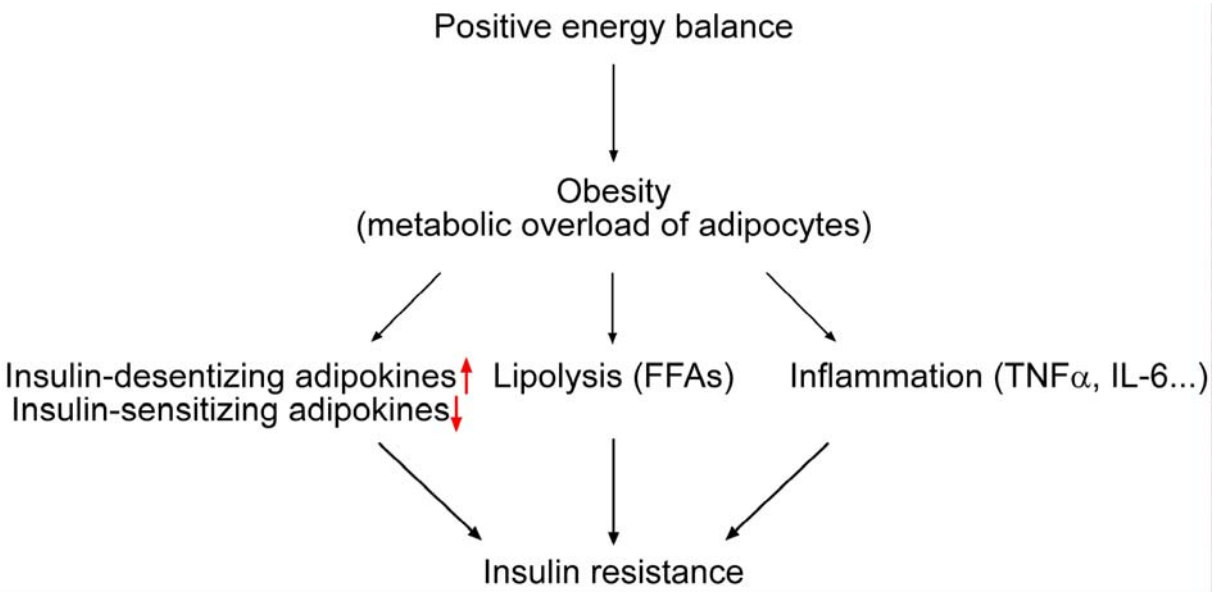

Fig. 1 Development of insulin resistance. Positive energy balance, caused by increase food intake and reduced energy expenditure, results in increase adiposity and obesity. Metabolically overloaded adipocytes secretes increase amount of insulin-desensitizing and reduced amount of insulin-sensitizing adipokines. Moreover, increase adiposity leads to the induction of inflammatory pathways and increase lipolytic activity. Increase levels of the inflammatory mediators (IL-6, TNFa), FFAs and insulin-desensitizing hormones results in the systemic insulin resistance.

4.3. Pancreatic β cell function

Insulin is secreted from pancreatic β cells in response to glucose. Several factors amplifying glucose-stimulated insulin secretion have been identified including insulinotropic gut peptides such as glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)[75], β-adrenergic stimulation[1] and neurotransmitters such as acetylcholine[76].

Secretion of insulin is regulated by a number of subsequent steps. Glucose enters pancreatic β-cells through the high affinity glucose transporter 2 (Glut-2). Glucose is metabolized through glycolysis followed by oxidative metabolism of glucose in the mitochondria leading to the production of ATP. As a consequence, the ATP/ADP ratio increases resulting in closure of ATP-dependent potassium channels and depolarization of the plasma membrane. This causes opening of voltage-gated calcium channels and extracellular calcium influx into the cell interior and finally leads to insulin granule exocytosis[1]. In fact, insulin granules within the cell undergo extensive movement. Insulin granules in β cells exist in two pools; vesicles which are morphologically docked to the plasma membrane, also termed the rapidly releasable pool, which accounts for approximately 10 % of the total cellular insulin content and vesicles located more far away from the plasma membrane, the so called intracellular reserve pool. The first phase of insulin secretion is mainly triggered by the rapidly releasable pool. In order to allow efficient insulin exocytosis during the second phase of insulin secretion, insulin granules from the reserve pool are transported to the proximity of the plasma membrane in a cytoskeleton-mediated, ATP-dependent manner[77].

As already mentioned above, insulin secretion is regulated also by insulinotropic agents; GLP-1 or β-adrenergic stimulation leads to an increase of cyclic AMP levels, which activates protein kinase A (PKA) and the guanine nucleotide exchange factor (EPAC2) and stimulating the closure of ATP-dependent potassium channels and release of intracellular calcium stores[1,

[75]. Acetylcholine released from parasympathetic synapses act through the muscarinic receptors and stimulate intracellular diacylglycerol (DAG) production, which also releases calcium from intracellular stores and promotes insulin granule secretion[78, 79].

4.4. Pancreatic β cell function in type-2 diabetes

During the state of insulin resistance, an increased amount of insulin is required for maintaining normal glucose levels. In general, full-blown type-2 diabetes occurs when the amount of insulin does not meet the metabolic demand and fasting glucose are above the normal values[15].

Hyperinsulinemia during the early phase of insulin resistance results from an increase in pancreatic β cell mass and from enhanced capacity of cells to secrete insulin. Pancreatic β cells expansion has been observed in insulin resistant, obese rats, mice as well as humans[80-82]. β cell expansion is caused by proliferation of existing β cells and/or neogenesis from pancreatic ductal cells[83]. It is not entirely clear what the relative contribution of these two processes is to compensate insulin resistance. However recent studies seem to confirm the presence of multipotent progenitor cells in pancreas from adult mice which can differentiate into functional β cells[84].

Several factors, which are increased in the serum of the insulin-resistant individuals, have been proposed to stimulate pancreatic β cell expansion. Especially increased levels of nutrients such as glucose and free fatty acids (FFA) can promote proliferation of pancreatic β cells[85, 86]. Moreover, multiple hormones including GLP-1, GIP, hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1) and insulin itself have been shown to stimulate pancreatic β cell proliferation[75, 87-89]. GLP-1 has been shown to promote pancreatic β cell proliferation, neogenesis and survival[75, 89]. Treatment of diabetic individuals with GLP-1 analogues ameliorates hyperglycemia and increases pancreatic β cell mass[67]. At the molecular level, signaling from GLP-1 receptor (GLP-1R) activates multiple signaling cascades including mitogen activated protein kinase (MAPK), PI3-kinase, and protein kinase A (PKA) and leads to activation of a transcriptional response including activation of pancreatic and

duodenal homeobox factor-1 (PDX-1), a crucial regulator of differentiation and function of pancreatic β cell[52]. In fact, effects of GLP-1 was shown to be completely abolished in the absence of PDX-1[90]. Another closely related incretin hormone, GIP, has been shown to promote similar effects on β cells, however the molecular mechanisms of GIP action are less understood[52, 67]. Interestingly, signaling mediated by insulin and IGF-1 has been shown to be crucial for maintaining an appropriate mass of pancreatic β cells in the body. Pancreatic β cell specific deletion of the insulin receptor in mice led to a reduced insulin content in the pancreas due to decreased pastnatal β cell growth and resulted in the development of diabetes[91, 92]. Consistently, IRS-2 has been shown to be crucial for β cell development and to compensate for peripheral insulin resistance, since IRS-2 knockout mice displayed loss of pancreatic β cells associated with decreased insulin secretion and glucose intolerance[93]. In contrast, IRS-1 rather seems to be important in mediating peripheral insulin action[93]. IRS-1 knockout mice display primarily severe insulin resistance but show pancreatic β cell hyperplasia to counteract insulin resistance[94]. Mice with deletion of the IGF-1 receptor in pancreatic β cells show decreased β cell mass and inappropriate development of pancreatic islets[93].

As mentioned above, pancreatic β cells can compensate peripheral insulin resistance not only through expansion of their mass, but also by increasing the capacity of the existing β cells to secrete insulin. In fact, several factors which were shown to promote pancreatic β cell expansion are also implicated in stimulation of insulin secretion. Increased pancreatic β cell glucose metabolism, stimulation with FFA, increased signaling by incretin hormones and parasympathetic nervous stimulation are the main factors accounting for the stimulation of β cell function during the state of insulin resistance[83]. Enhanced glucose metabolism is coupled to increased expression of the rate limiting factors involved in this process. Increased glucose entry into the pancreatic β cells is mediated by the glucose transporter GLUT-2, which was

shown to be more abundant on the surface of β cells from insulin resistant subjects[83]. Similarly, enhanced entry of glucose into the Krebs cycle in mitochondria is coupled to increased activity of glucokinase, the rate limiting enzyme in cytosolic glycolysis[83]. Stimulation of insulin secretion by FFAs, the levels of which are markedly increased in the state of obesity and peripheral insulin resistance, is mediated by G protein-coupled receptor 40 (GPR40). Signaling from this receptor leads to increased intracellular Ca^{2+} content and stimulates insulin granule exocytosis[95, 96]. Also the incretin hormone GLP-1 has been shown to promote pleiotropic effects on β cell function. Enhanced signaling from the GLP-1 receptor leads to the stimulation of insulin biosynthesis, promotes the release of intracellular Ca^{2+} stores and evokes depolarization of the cell membrane by promoting closure of the ATP-dependent potassium channel[1, 75, 97]. Several lines of evidence implicated the autonomic nervous system in the regulation of insulin secretion. In general, parasympathetic neurons stimulate and sympathetic neurons suppress insulin secretion[98]. Recent results indicated that parasympathetic neurons might contribute to enhanced insulin secretion in the state of insulin resistance since overexpression of the muscarinic M3 receptor (receptor for acetylcholine, one of the main neurotransmitter of the parasympathetic neurons) specifically in the pancreatic β cells leads to increased insulin release, improved glucose tolerance and protects mice from diet-induced glucose intolerance and hyperglycemia. Conversely, deletion of muscarinic M3 receptor specifically in β cells results in impaired insulin release and glucose intolerance[79].

Although pancreatic β cells initially can compensate for an increased insulin demand caused by peripheral insulin resistance, several initial compensatory mechanisms in the long term lead to β cell failure[15, 83] (Fig. 2). Among others, chronic exposure of pancreatic β cells to high glucose and high levels of FFA, resulting in so called gluco- and lipotoxicity, are most prominent factors contributing to the β cell failure[99, 100]. Gluco- and lipotoxicity is manifested by a decreased pancreatic β cell function caused by a reduction in insulin transcription and

translation, decreased expression of proteins involved in regulation of glucose-stimulated insulin secretion such as GLUT-2 and voltage-gated Ca^{2+} channel. Moreover, chronic exposure to both glucose and FFA results in the induction of ER- and oxidative stress, which might in turn lead to apoptosis[99-101]. Along this line, exposure of β cells with high glucose results in activation of sterol regulatory element binding protein-1c (SREBP-1c) and subsequent induction of lipogenic pathways, which results in the accumulation of lipids in β cells and induction of ER-stress[90]. In addition to the induction of ER-stress by deposition of lipids in β cells, FFA mediated signaling through the β cell-specific receptor GPR40 also contributes to β cell failure during development of diabetes, since overexpression of this receptor in β cells leads to failure of these cells and the development of diabetes[96]. Hyperglycemia has been also implicated in the stimulation of mitochondrial superoxide production and induction of oxidative stress[102]. Oxidative stress contributes to β cell failure by several mechanisms including induction of apoptosis[100] and by decreasing the production of ATP through activation of uncoupling protein 2 (UCP2), leading to decreased insulin exocytosis[103]. Another possible mechanism mediating the loss of β cell mass is deposition of islet amyloid in β cells. Formation of amyloid deposits results from aggregation of islet amyloid polypeptide (IAPP). IAPP is secreted from β cells together with insulin; moreover secretion of these proteins seems to be co-regulated[104]. Interestingly, IAPP aggregation does not occur in rodents due to differences in the amino acid sequence between human and rodent IAPP. In fact, the only genetic evidence highlighting the importance of pancreatic β cell amyloid deposition in type-2 diabetes stems from experiments in which ectopic expression of the human IAPP specifically in mouse pancreatic β cells has been achieved. Overexpression of human IAPP in wild-type mice did not result in alterations of β cell function and deposition of amyloid aggregates. However, in the insulin resistant ob/ob mouse strain ectopic

expression of human IAPP resulted in severe amyloid deposition, loss of β cells and severely increased hyperglycemia[105].

Apart from above described mechanisms, a number of monogenic mutations leading to β cell failure and diabetes have been described. The most characterized are a group of mutations collectively called maturity onset diabetes of the young (MODY). MODY mutations cause approximately 2-5% of all cases of diabetes. Six different subtypes of MODY mutations are classified, based on the affected locus that has been identified (MODY1 – MODY6)[106]. The most common form, MODY2 results from the mutation in the gene encoding glucokinase: the enzyme responsible for the initial step of glycolysis. MODY2 mutations result in defective glucose sensing in pancreatic β cell and increases the threshold of glucose concentration required for initiation of insulin secretion[107]. MODY3 results from the mutations in hepatocyte nuclear factor 1α (HNF1α); usually subjects with mutations in this gene develop severe diabetes caused by insufficient insulin secretion[108]. Other mutations in the transcription factors hepatocyte nuclear factor 4α (HNF4α) and hepatocyte nuclear factor 1β (HNF1β) have been identified as MODY1 and MODY5, respectively[109, 110]. Mutations in the key transcription factor PDX-1, involved in differentiation and maintenance of pancreatic β cell function, has been described as MODY4[111]. MODY6 results from mutations in NeuroD1/β2 and leads to the development of severe diabetes[112].

In conclusion, pancreatic β cell failure strongly contributes to the development of type-2 diabetes mellitus. As for many other compensatory mechanisms, factors and pathways initially enhancing the efficiency of β cells, in the same time may lead to induction of maladaptive processes accounting for β cell failure when they are chronic in nature (Fig. 3).

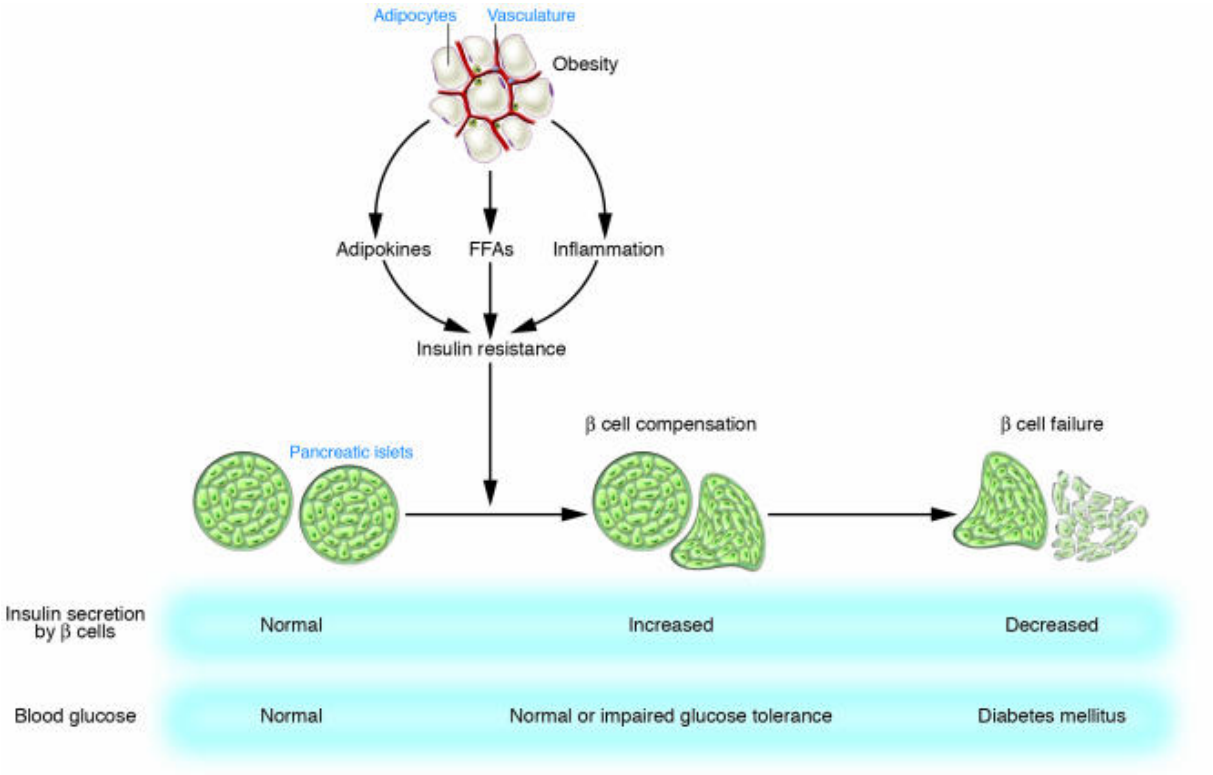

Fig. 2 Development of type-2 diabetes. Increased adiposity in obese subjects leads to increased circulating adipokines, FFAs and inflammatory mediators resulting in peripheral insulin resistance. Increased demands of insulin caused by insulin resistance is initially compensated by pancreatic β cells through different mechanisms, which initially prevents development of full-blown type-2 diabetes mellitus. However, prolonged exposure of cells to these factors, which are also key in inducing insulin resistance, lead to pancreatic β cell failure and results in type-2 diabetes mellitus (adapted from [15]).

4.5. The Mitogen-Activated Protein Kinase (MAPK) signaling cascade

A number of stress-induced signaling pathways including MAPKs have been shown to be implicated in the development of insulin resistance, pancreatic β cell dysfunction and as consequence in the development of type-2 diabetes[4-8, 19, 20, 58, 65, 67].

In mammals, four conventional and three atypical MAPK families exist. The conventional MAPK are comprised by the extracellular regulated kinase 1 and 2 (ERK1,2), c-jun N-terminal kinase (JNK), p38 and the extracellular regulated kinase 5 (ERK5). The atypical MAPKs consist of the extracellular regulated kinase 3 and 4 (ERK3,4), the extracellular regulated kinase 7 and 8 (ERK7,8) and the NEMO-like kinase (NLK) [113-115]. Within the kinase domain, all the members of the MAPK branch display more than 40% of amino acid sequence identity compared to ERK1[115].

Conventional MAPKs are activated by a largely defined upstream protein kinase cascade. In general, upon stimulation with multiple agents such as growth factors, cytokines and stress, G-protein coupled receptors activate MAPK kinase kinases (MAPKKKs), which target downstream MAPK kinases (MAPKKs) leading to subsequent activation of MAPKs[113]. Another characteristic feature of the conventional MAPK is the presence of the Thr-X-Tyr motif in the activation loop, which is the site of activation by MAPKK[60]. The activation of atypical MAPKs is generally not mediated by MAPKKs, although the exact mechanisms of activation of these kinases are still not well understood[115].

Through phosphorylation of downstream targets, MAPK regulates a whole plethora of basic cellular processes including proliferation, differentiation, apoptosis, migration and inflammation[60, 113, 116] (Fig. 3).

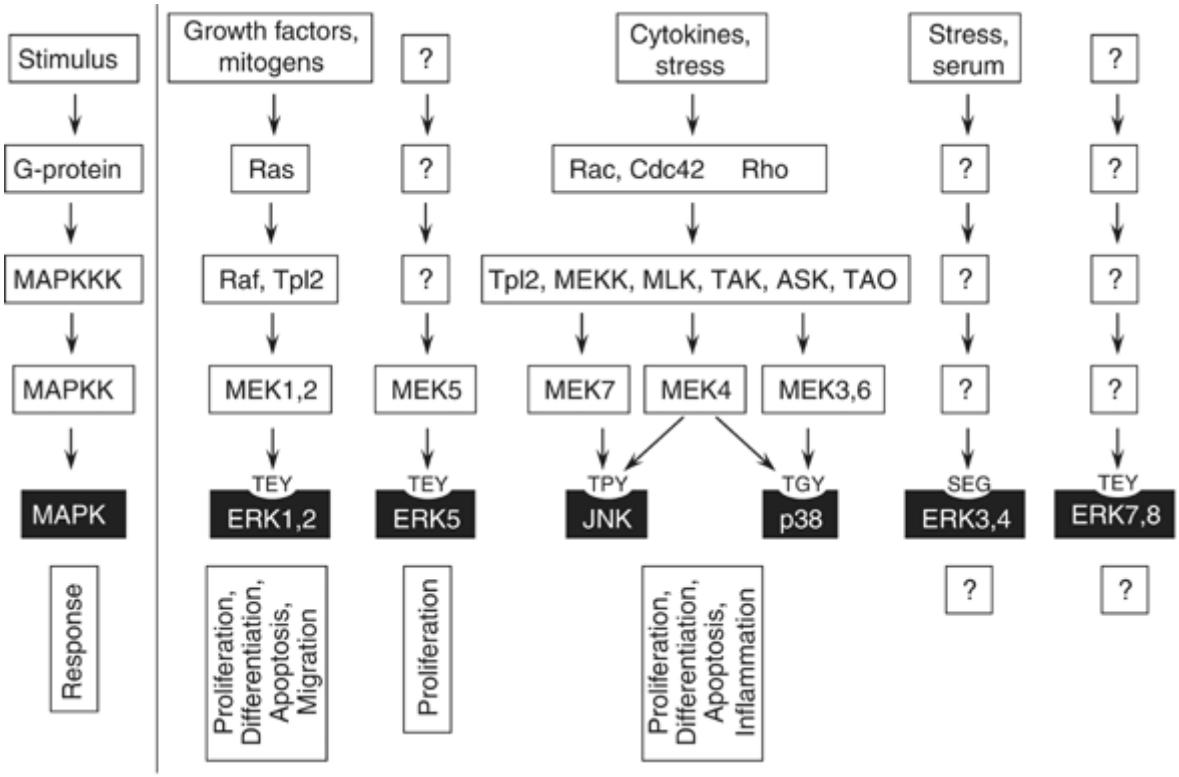

Fig. 3 Stress-activated MAPK signaling modules. MAPK regulates most of the basic cellular processes. MAPK are comprised of a three-tier kinase module, in which a MAPK is activated upon phosphorylation by MAPKK, which in turn is activated when phosphorylated by MAPKKK (adapted from[113]).

4.6. The MAPK family JNK

The JNK proteins are encoded by three genes: JNK1, JNK2 and JNK3. While JNK1 and JNK2 are expressed ubiquitously, JNK3 has a limited pattern of expression and is largely restricted to brain, heart, and testis[117]. Numerous studies in animal models established a key role of JNK in various pathological conditions including cancer, cardiac hypertrophy/failure, neurodegenerative disorders, arthritis and asthma. Lately, it has been demonstrated that JNK1 but not JNK2 promotes obesity and insulin resistance[4]. However, very recent data from the same research group provide evidence that JNK2 is also involved in metabolic regulation, but its function is compensated by JNK1[59]. At the molecular level, JNK directly suppresses insulin signaling through a phosphorylation-induced inactivation of the insulin receptor substrate 1 (IRS-1) at Ser307[60]. Activation of JNK and other inflammatory and/or stress-induced kinases including inhibitor of nuclear factor-κB (IKK) most likely occurs through metabolic overload-mediated induction of ER stress[54]. ER stress-mediated activation of these kinases leads to an enhanced inflammatory response since the very same kinases are key mediators of transcription and secretion of numerous cytokines. The activity of these kinases is further enhanced in response to elevated cytokines and hence exacerbates insulin resistance. For example, TNFα, a pivotal pro-inflammatory cytokine, is highly expressed in adipose tissue of obese and insulin resistant animals and humans[42, 118]. Concordantly, obese mice lacking either TNF-α or TNF-α receptors are protected against insulin resistance[4]. Recent studies also support that increased activity of JNK attenuates insulin production and secretion as well as increases apoptosis in the pancreatic islets[119-125].

ER stress induction and subsequent activation of JNK also occurs in cholesterol-overloaded macrophages in the atherosclerotic lesion[90]. In our own previous study, we demonstrated pronounced activation of JNK in atherosclerotic lesions in mice. Moreover, we provided clear

in vivo evidence that JNK2 promotes atherosclerosis through a macrophage-specific mechanism[61].

These mechanisms are likely to occur not only in adipocytes and macrophages but also in other cell types including hepatocytes, skeletal muscle cells and β-cells rendering these kinases as very attractive drug targets in type-2 diabetes. Thus, ER stress and JNK activation represent key unifying mechanisms in the development of the various conditions within the metabolic syndrome.

4.7. The MAPK family p38

The closely related p38 signaling cascade likewise appears to mediate various molecular events in response to inflammatory, stress and metabolic stimuli. Four isoforms of p38 MAPK have been identified: p38α[126], p38β [127], p38γ [128] and p38δ[129]. p38 isoforms share about 60% homology, the highest between the α and β isoforms (74%).

Activation of p38 occurs via dual phosphorylation of conserved TGY motifs by the MAPKKs, MKK6 and MKK3[117]. All the isoforms share two highly similar common docking domains predominantly responsible for the kinase substrate interaction. In contrast to other isoforms, p38γ contains a PDZ binding motif at the C-terminal end, allowing interaction with PDZ domains of other proteins. Despite the high similarity between the four isoforms, the N- and C-terminal sequences of the amino acids display a high variability and might determine specific functions of these proteins.

In contrast to JNK, very little is known about the role of p38 *in vivo* and the contribution of different p38 isoforms to several biological processes is poorly understood, and in some instances even controversial. This is mainly due to the fact that isoform-specific inhibitors start to be available only now. In addition, conventional knockout, as well as conditional knockout mice have been generated only very recently and the analyses of these animals have just started.

p38 has been mainly implicated in the regulation of innate immunity. Several studies have suggested an involvement of p38 in the production of inflammatory factors such as TNFα, IL-1[126, 130, 131], IL-6[132], IFN-γ[133, 134] and IL-12[134, 135], but also the anti-inflammatory factor IL-10[134, 136, 137]. Expression studies revealed that p38 might be involved in inflammatory diseases such as rheumatoid arthritis[138], Alzheimer's disease[53], inflammatory bowel disease[139] and acute respiratory distress syndrome[140].

Fig. 3 Alignment of p38 isoforms. Sequence alignment of different p38 isoforms revealed their high homology (around 60%). The lowest similarity is observed at the ends of the sequence. All the isoforms share the dual phosphorylation activatory motif (blue frame) and the common docking domain (yellow frame). The PDZ domain binding motif is unique to the p38γ isoform (violet frame). In the right panel, the homology tree of p38 isoforms is indicated

However, the role of p38 is not confined to cytokine production. Several experiments support the idea that p38 might play a central role in cellular metabolic processes. With respect to adipocytes and muscle, p38 has been reported to be activated in insulin-resistant peripheral tissues from diabetes patients[141]. Insulin-stimulated activation of p38 has been observed in rat skeletal muscles[142], mouse brown adipocytes[143], murine 3T3-L1 adipocytes[144-146] and L6 muscle cells[147, 148]. However, an involvement of p38 in glucose uptake through direct action on GLUT4, the main glucose transporter in muscle cells and adipocytes, remains highly controversial at this point[149-151]. Nevertheless, central metabolic regulators such as the transcription factor CREB[152, 153] and the transcriptional cofactor PGC-1α[154] are targets of p38 implicating a role of this kinase family in thermogenesis, gluconeogenesis and lipid metabolism[154-158]. The transcriptional coactivator PGC-1α coordinates expression of genes including UCP-1 stimulating mitochondrial oxidative metabolism[29]. p38 also directly stimulates expression of the UCP-1 gene through phosphorylation of the transcription factor ATF-2[73]. Moreover, p38 might be implicated in regulation of insulin production and pancreatic β-cell biology[159, 160].

Finally, data *in vitro* have demonstrated that p38 activation upon insulin, TNFα, free fatty acids and other stress stimuli impairs insulin signaling in adipocytes and skeletal muscle cells through very similar mechanisms as described above for JNK[161]. Furthermore, there is also experimental evidence *in vitro* that p38 might promote adipogenesis[40].

With respect to the cardiovascular part of the metabolic syndrome, several independent studies imply that p38 also partially regulates initial events in atherosclerosis such as endothelial cell activation, leukocyte adhesion/migration and foam cell formation[162-171]. Accumulation of free cholesterol is known to occur in macrophages at later stages of atherosclerosis and is associated with ER stress and increased apoptosis leading to secondary tissue necrosis and destabilization of the atherosclerotic plaque. Indeed, p38 in cooperation with

JNK seems to convert ER stress into the expression of cytokines in macrophages[65]. On the other hand, novel data support the idea that p38 cooperates with the NF-κB pathway to mediate macrophage apoptosis[155] (Fig. 4).

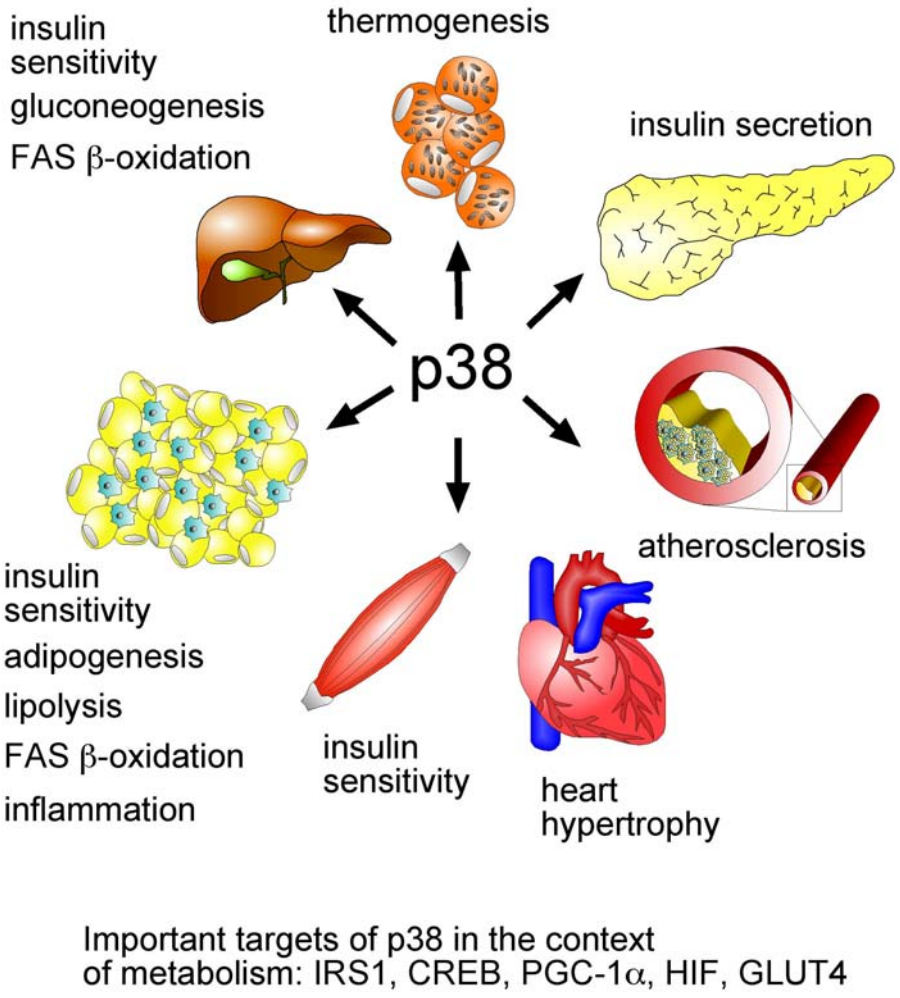

Fig. 4 An overview of possible functions of p38 isoforms in different metabolic processes. Multiple *in vitro* and expression/activation studies reveled possible functions of p38 in metabolism and functions of different cell types and organs (for details: see text)**.**

However, the requirement of p38 *in vivo* in most of the processes mentioned above, as well as the specific roles of p38 isoforms are largely unknown. This is mainly due the fact that most of the available data about p38 and its role in different biological processes were obtained by using pyridynyl imidazole-based p38 inhibitors, which target p38α and p38β, but not p38γ and p38δ[172-175].

Only recently, investigators start to use isoform-specific approaches (knockout mice) highlighting that different isoforms of p38 indeed possess different functions.

The best characterized p38 isoform is p38α. It has been shown that p38α knockout mice die at midgestation, likely due to a defective erythropoiesis[29]. However, another research team, using tetraploid blastocyst injection approach, demonstrated that p38α is required for placental organogenesis suggesting that all other observed developmental defects are most likely secondary[176]. Recently another group using conditional p38α knockout mice showed that p38α is not required for erythropoiesis since deletion of p38α in the embryo, but not in the extraembryonic tissue, using the More-Cre deleter strain, does not result in embryonic mortality, but results in postnatal mortality due to severe lung dysfunction[177]. One of the first sets of data concerning the function of the p38α in adult mice using a conditional approach has been obtained only very recently. p38α seems to play a critical role in cardiomyocyte survival and proliferation but not in cardiac hypertrophic growth[178, 179]. Other recent reports suggested that p38α is a fundamental regulator of proliferation and carcinogenesis *in vivo*[177, 180-182] (Fig. 6).

p38α shares more than 70% amino acid sequence identity with p38β. Thus, inhibitors and/or deletion strategies used in most studies described above targeted both p38α and p38β isoforms. Nevertheless, it was assumed that p38α and p38β cooperate in inflammatory and metabolic processes. This assumption was lately contradicted, since it has been demonstrated that p38β-specific knockout mice showed no differences in several *in vivo* and *in*

vitro models of inflammation [151]. Furthermore, another recent study does not support a direct link between p38β and insulin signaling [151]. However, another group showed that p38β might be involved in regulation of cardiomyocyte hypertrophy [183], suggesting specific functions of this isoform.

The least characterized isoforms are p38γ and p38δ. p38γ shares 60% homology to p38α. So far *in vitro* data suggests that this isoform might be involved to regulate the osmotic stress program [184] and regulates glucose uptake in skeletal muscles[185] (Fig. 5).

The fourth isoform of p38 MAPK kinase – p38δ, also known as SAPK4, has been cloned and partially characterized in 1997. p38δ shares significant homology with other p38 isoforms (60%) and about 40% of homology to other members of the MAPKs, JNK and ERK[129].

Recent *in vitro* studies have shown that p38δ might be involved in keratinocyte differentiation[186-188] and PKCδ-dependent keratinocyte apoptosis[188]. p38δ could also be involved in the progression of neurodegenerative disorders, since it has been shown to phosphorylate Tau, a microtubule-associated protein that is abnormally hyperphosphorylated in the filamentous lesions that define a number of neurodegenerative diseases collectively referred to as tauopathies[189, 190]. Moreover, p38δ might mediate carcinogenesis by affecting survival, proliferation and invasion of the tumor cells[191]. Several other *in vitro* studies point out that p38δ might be important in the regulation of transcription, translation and activation of different proteins. p38δ has been shown to phosphorylate and deactivate eukariotic elongation factor 2 kinase (eEF2 kinase) leading to dephosphorylation and activation of eukariotic elongation factor 2 (eEF2)[192]. p38δ also seems to suppress MKK6-mediated AP-1 activation through downregulation of c-jun and c-fos expression[193]. Furthermore, it appears to be required for transforming growth factor β 1 (TGFβ1)-stimulated vascular endothelial growth factor 164 (VEGF 164) transcription in murine mesengelian cells[194] and last, but not

least suppresses LPS stimulated transcription of heme oxygenase 1 (HO-1)[195] (Fig. 5). But no specific *in vivo* functions for p38δ have been reported thus far.

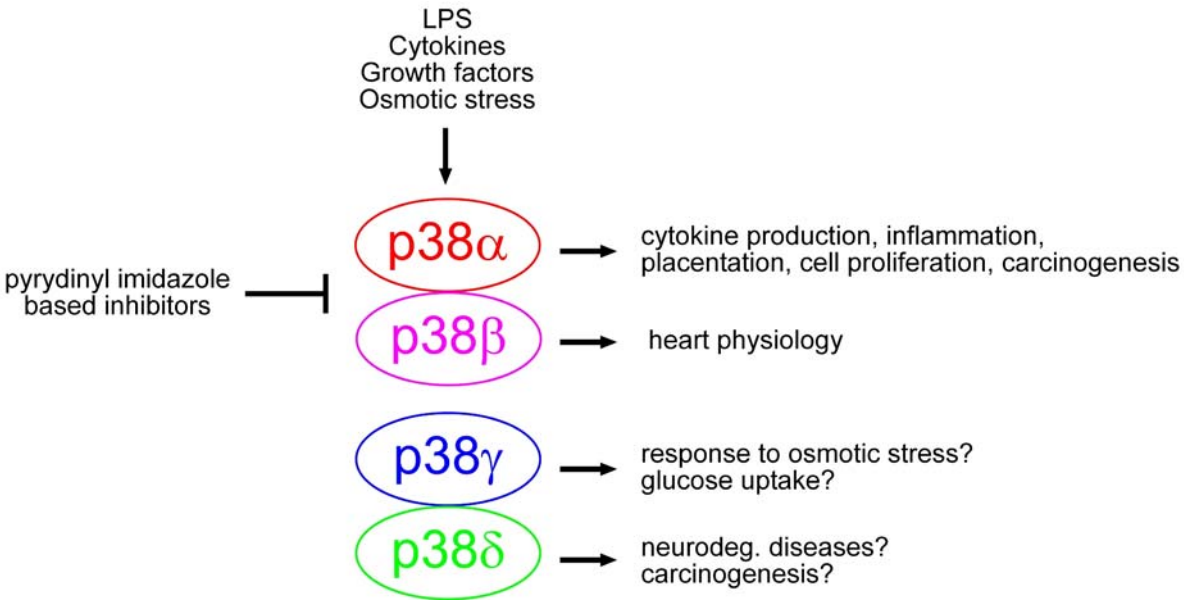

Fig. 5 Functions of p38 isoforms. All four p38 isoforms have been shown to be activated by similar stimuli, however due to the structural differences, pyrydinyl imidazole-based inhibitors block the activity only of the two closest related isoforms α and β, but not γ and δ. Analyzes of the isoform-specific functions revealed several functional differences (for details: see text).

5. Aim of the project

Over the last decades, the prevalence of type-2 diabetes and associated disorders increased dramatically[196]. Multiple studies established the function of stress-activated signaling cascades including MAPKs in a number of pathological conditions including type II diabetes and associated disorders[4, 19, 54, 61, 65, 197-202].

In particular, also the stress kinases p38 have been implicated in multiple pathological conditions including diseases related to metabolism. However, most of the conclusions were based on *in vitro* and expression studies. Moreover, specific functions of the p38 isoforms have not been identified so far. The aim of my project was to identify a specific *in vivo* function of the fourth p38 isoform, p38δ in particular in the context of metabolism.

6. Results

The p38 MAPK family consists of the four genes p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12) and p38δ (MAPK13). p38α has been explored in a plethora of signalling cascades and in the context of several diseases[9]. In contrast, *in vivo* functions of the other p38 isoforms are largely unknown. The isoform p38δ shares around 60% homology with the other p38 family members and about 40% homology with other MAPKs. Alike p38α, p38δ is activated by various stress stimuli, including inflammatory cytokines[174]. Recent *in vitro* studies have demonstrated that p38δ might be involved in keratinocyte differentiation[187] and PKCδ-dependent keratinocyte apoptosis[188], as well as the progression of neurodegenerative disorders referred to as tauopathies[190] but no specific *in vivo* functions have been reported thus far.

To address such functions, we have generated *Mapk13* floxed mice and crossed them with mice expressing Cre recombinase under control of the *Protamine* promoter. The *Protamine* promoter-driven Cre recombinase efficiently recombines target sequences in the male germ line of mice[203]. With this approach, we obtained *Mapk13* null mice (*Mapk13*$^{\Delta/\Delta}$ mice) and corresponding wild type control littermates (*Mapk13*$^{+/+}$) (Fig. 6a, b and c). The *Mapk13*$^{\Delta/\Delta}$ mice did not show any apparent macro- and histopathologic abnormalities (data not shown). The highest expression of *Mapk13* mRNA has been reported in mouse pancreas, testis, small intestine and prostate gland[129]. We now document that in the pancreas p38δ is abundantly expressed also at the protein level, and in similar amounts in the exocrine and endocrine parts. In contrast, no measurable expression of p38δ could be detected in the main insulin-sensitive organs, including adipose tissue, liver, and skeletal muscle (Fig. 7a and supplementary Fig. 8a).

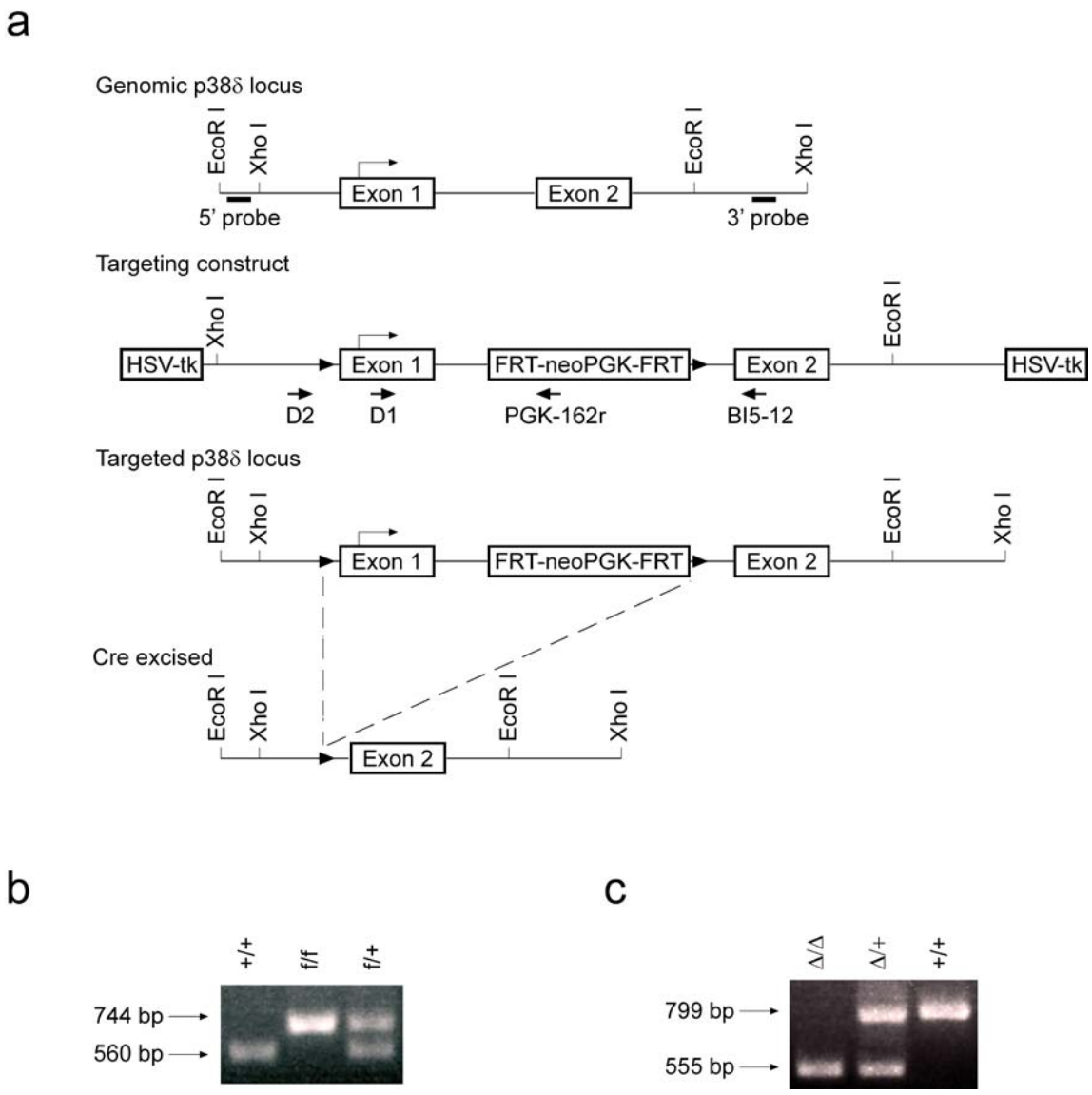

Figure 6 Generation of *Mapk13* floxed mice and *Mapk13*$^{\Delta/\Delta}$ mice using *Protamine-cre*. (a) Schematic representation of the targeting strategy employed to generate a floxed allele of *Mapk13*. Exons 1 and 2 of the *Mapk13* open reading frame are represented by rectangles, thin lines represent untranslated regions of the *Mapk13* locus. The neomycin resistance gene (for positive selection) flanked by two FRT sites (FRT-neoPGK-FRT) and the HSV-tk cassettes (for negative selection) are indicated. The arrows indicate the position of the primers for genotyping, loxP sites are shown as triangles. The strategy for generation of the targeted allele is described in Methods. **(b)** PCR analysis of genomic DNA isolated from adult *Mapk13*$^{+/+}$, *Mapk13*$^{f/+}$ and *Mapk13*$^{f/f}$ mice using D1, PGK-162r and BI5-12 primers, yielded a 744 bp band corresponding to the floxed allele and a 560 bp band corresponding to the wild type allele. **(c)** PCR analysis of genomic DNA isolated from

mice carrying deletion of *Mapk13* by Protamine Cre; *Mapk13*$^{\Delta/\Delta}$, *Mapk13*$^{\Delta/+}$ and *Mapk13*$^{+/+}$. PCR was performed using D2 and BI5-12 and yielded a 799 bp band corresponding to the wild type allele and a 555 bp band corresponding to the deleted *Mapk13* allele.

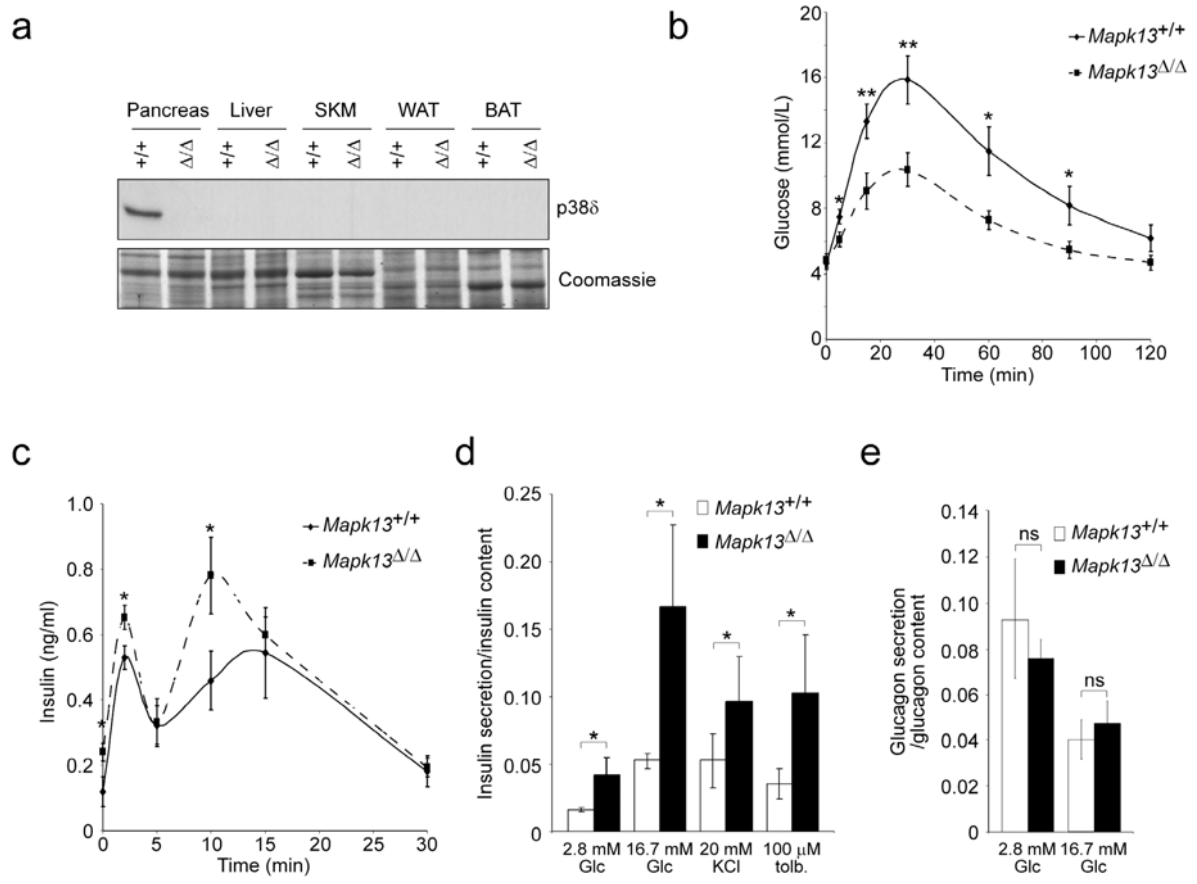

Figure 7 Mice lacking *Mapk13* show improved glucose tolerance and enhanced insulin secretion. (a) Western blotting revealed expression of p38δ in wild type (+/+) pancreas but not in liver, skeletal muscle (SKM), white (WAT) and brown adipose tissue (BAT) as well as tissues from *Mapk13* null (Δ/Δ) mice. Coomassie blue was used to confirm equal loading. **(b)** Glucose tolerance of *Mapk13* mice (squares and dashed line, n=10) was significantly improved (*****p<0.05, **p<0.01) compared to that of wild type *Mapk13*$^{+/+}$ littermates (diamonds and solid line, n=9). **(c)** Parallel measurements of serum insulin showed that, during the GTT test, *Mapk13*$^{\Delta/\Delta}$ mice (squares and dashed line, n=5 for each time point) had higher (*****p<0.05) basal and stimulated levels of the circulating hormone than *Mapk13*$^{+/+}$ mice (diamonds and solid line, n=5 for each time point). **(d)** In response to indicated stimuli (Glc: glucose, KCl: potassium chloride, tolb.: tolbutamide), insulin release was enhanced in isolated *Mapk13*$^{\Delta/\Delta}$ islets (black bars, n=15) compared to *Mapk13*$^{+/+}$ islets (white bars, n=15)

(*p<0.05). **(e)** Glucagon secretion in *in vitro* cultured isolated islets in response to 2.8 mM and 16.7 mM Glucose. Under both conditions no differences in islets isolated from *Mapk13*$^{\Delta/\Delta}$ (black bars, n=14) compared to *Mapk13*$^{+/+}$ (white bars, n=16) could be observed. Glucagon secretion was normalized to glucagon content.

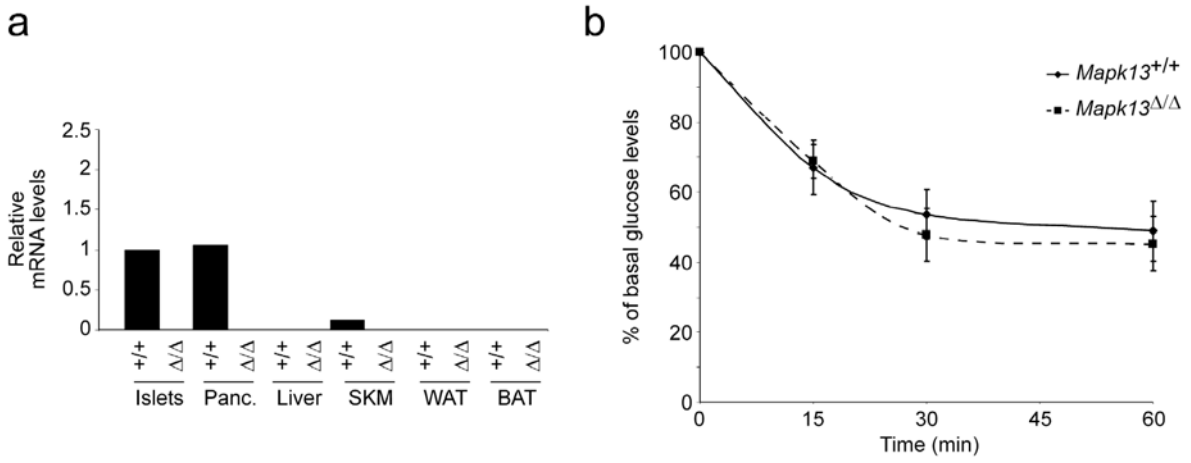

Figure 8 Abundant expression of p38δ in pancreas and normal insulin sensitivity in *Mapk13*$^{\Delta/\Delta}$ mice. (a) Relative expression of *Mapk13* mRNA, as tested by quantitative real time (RT)-PCR, showed expression of p38δ in isolated islets and total pancreas (Panc.) of *Mapk13*$^{+/+}$ but not *Mapk13*$^{\Delta/\Delta}$ mice, and a low expression in skeletal muscle (SKM) but not in liver, white (WAT) and brown adipose tissue (BAT). Expression levels were normalized to those of *18S* rRNA. **(b)** Insulin tolerance tests. Insulin (1 U/kg) was injected intraperitoneally in ad libitum fed mice and glucose was measured at indicated time points. Insulin sensitivity was equal in *Mapk13*$^{\Delta/\Delta}$ (squares and dotted line, n=5) and *Mapk13*$^{+/+}$ (diamonds and solid line, n=6) mice. No signifcant differences in insulin sensitivity between *Mapk13*$^{\Delta/\Delta}$ and *Mapk13*$^{+/+}$ mice were observed.

This expression pattern prompted us to investigate the role of p38δ in pancreatic β cells and glucose homeostasis. For this purpose, we challenged mice with intraperitoneal glucose and monitored blood glucose levels over time (glucose tolerance test, GTT). *Mapk13*$^{\Delta/\Delta}$ mice fasted for 16 hours showed a significantly enhanced glucose tolerance compared to *Mapk13*$^{+/+}$ mice (Fig. 8b), suggesting increased peripheral insulin sensitivity

and/or enhanced insulin release. In response to administration of insulin (insulin tolerance test, ITT), plasma glucose levels similarly declined in *Mapk13*$^{\Delta/\Delta}$ and *Mapk13*$^{+/+}$ mice indicating normal insulin sensitivity (Fig. 8b). In contrast, *Mapk13*$^{\Delta/\Delta}$ mice displayed higher levels of circulating insulin than *Mapk13*$^{+/+}$ control mice during the glucose tolerance test (Fig. 7c), suggesting enhanced insulin secretion. Accordingly, islets isolated from *Mapk13*$^{\Delta/\Delta}$ mice showed a higher basal as well as glucose-stimulated insulin release than islets of *Mapk13*$^{+/+}$ littermates (Fig. 7d), while glucagon secretion was unaltered in *Mapk13*$^{\Delta/\Delta}$ compared to *Mapk13*$^{+/+}$ mice (Fig. 7e) indicating that the insulin-secreting pancreatic β cells but not the glucagon-producing α cells in islets are affected. To confirm that lack of p38δ enhances insulin secretion in pancreatic β cells, we generated MIN6 cells (murine insulinoma cell line) stably expressing small hairpins (shRNA) against *Mapk13*, and control cells expressing an empty vector. Efficient knockdown of p38δ was confirmed by Western blotting (Fig. 9a). Compared to control cells, MIN6 cells lacking *Mapk13* featured enhanced insulin secretion in the presence of basal (2.8 mM) but not of stimulatory glucose levels (25 mM), possibly because MIN6 cells are maximally stimulated at this glucose concentration (Fig. 9b).

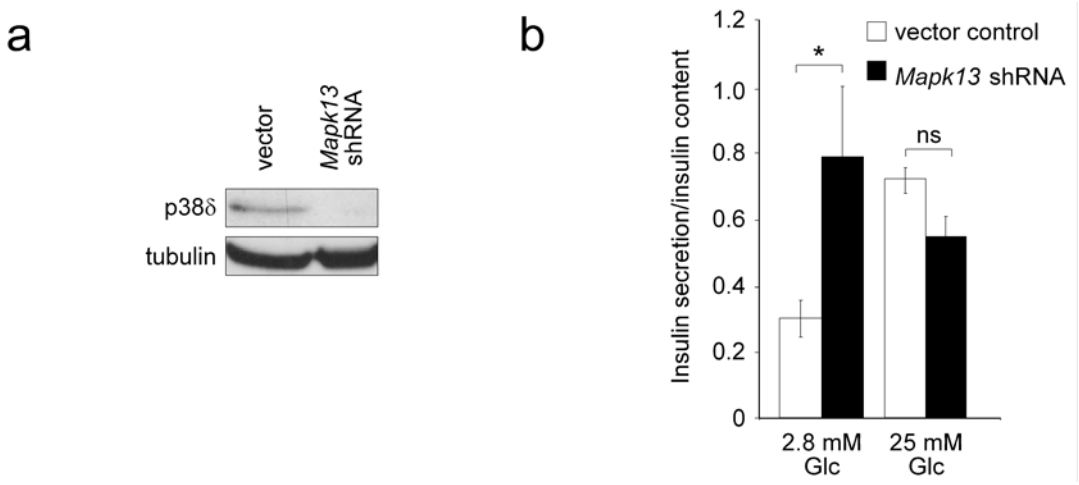

Figure 9 Stable expression of shRNA against *Mapk13 in* MIN6 cells enhances insulin secretion. (a) Western blot to determine endogenous expression of *Mapk13* in MIN6 cells stably expressing a control vector and small

hairpin RNA (shRNA) against *Mapk13*. Efficient deletion of *Mapk13* was achieved in shRNA-expressing cells. Tubulin was used as a loading control. **(b)** Insulin secretion of MIN6 cells stably expressing a shRNA against *Mapk13* and control cells expressing the empty vector. Compared to control cells (white bars), cells depleted of *Mapk13* (black bars) showed enhanced insulin secretion at basal (2.8 mM) but not at stimulatory glucose levels (25 mM) (*$p<0.05$ and ns, not significant, unpaired Student's t test).

To assess whether enhanced kinase activity of p38δ in pancreatic β cells was sufficient to suppress insulin secretion, we generated INS1 cells (rat insulinoma cell line) stably expressing hemagglutinin (HA), HA-tagged wild type *Mapk13* and HA-tagged constitutively active *Mapk13*. Constitutive p38δ activity was obtained by point mutations resulting in the substitution of aspartate 176 with alanin (D176A) and phenylalanine 324 with serine (F324S), as previously described[204]. We tested the activity of these mutants with an *in vitro* kinase assay using recombinant HIS-tagged ATF-2 as a substrate. Amino acid substitution F324S was more efficient at enhancing p38δ activity than substitution D176A (Fig. 10a) and was thus chosen for functional experiments. Stable expression of HA, HA-tagged wild type (HA-*Mapk13*^{wt}) and mutated HA-tagged *Mapk13* (HA- *Mapk13*^{F324S}) was confirmed by Western blotting (Fig. 10b). INS1 cells stably expressing wt p38δ revealed a glucose-induced insulin secretion similar to that of control cells (Supplementary Fig. 4c). In contrast, cells expressing the constitutively active p38δ displayed a significantly suppressed insulin secretion under both basal and stimulatory glucose conditions (Fig. 10c). These findings indicate that p38δ is a negative regulator of insulin secretion.

Enhanced insulin release as a result of *Mapk13* loss could be the consequence of an increase in the β cell mass, alterations in the pancreatic islet cell distribution and/or enhanced insulin production. Immunofluorescence analysis showed normal distribution of pancreatic β and α cells in both *Mapk13*$^{\Delta/\Delta}$ and *Mapk13*$^{+/+}$ mice (Fig. 11a). Quantification of the islet area

relative to the whole pancreatic area revealed no differences (Fig. 11b), and total pancreatic insulin content as well as total insulin content per islet were equal (Fig. 11c and d).

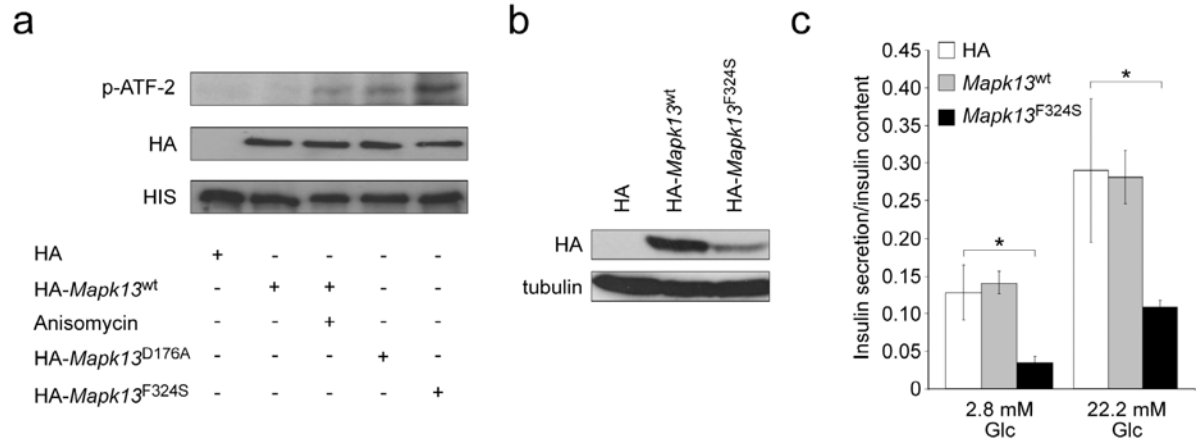

Figure 10 Ectopic expression of constitutive active p38δ suppresses insulin secretion in INS1 cells. (a) *In vitro* kinase assay using recombinant histidin (HIS)-tagged ATF-2 as a substrate of immunoprecipitated ectopic hemagglutinin (HA)-tagged p38δ. 293 cells were transfected with expression vectors containing HA (lane 1), wild type HA-*Mapk13* (HA-*Mapk13*^{wt}) without (lane 2) and with anisomycin (a known activator of p38δ) (lane 3), constitutive active HA-*Mapk13*^{D176A} (lane 4) and constitutive active HA-*Mapk13*^{F324S} (lane 5). After in vitro kinase reaction, proteins were separated by SDS-page. In vitro kinase activity was tested by measuring phosphorylation of ATF-2 (p-ATF-2) (upper lane). Equal loading of p38d and ATF-2 was confirmed using an HA antibody and a HIS antibody, respectively (middle and lower lanes). (b) Western blot to determine ectopic and stable expression of HA (lane 1), HA-p38δ^{wt} (lane 2) and HA-p38δ^{F324S} (lane 3) in INS1 cells. Tubulin was used as loading control. (c) Insulin secretion in INS1 cells stably expressing hemagglutinin (HA) tag, HA-tagged wt p38δ (p38δ^{wt}) and constitutive active p38δ (p38δ^{F324S}). p38δ^{F324S} expressing cells (black bars) showed reduced insulin secretion under both basal and stimulatory glucose levels (*$p<0.05$, unpaired Student's t test), compared to cells expressing either p38δ^{wt} (grey bars) or HA (white bars). In all experiments, insulin secretion was normalized to total cellular insulin content.

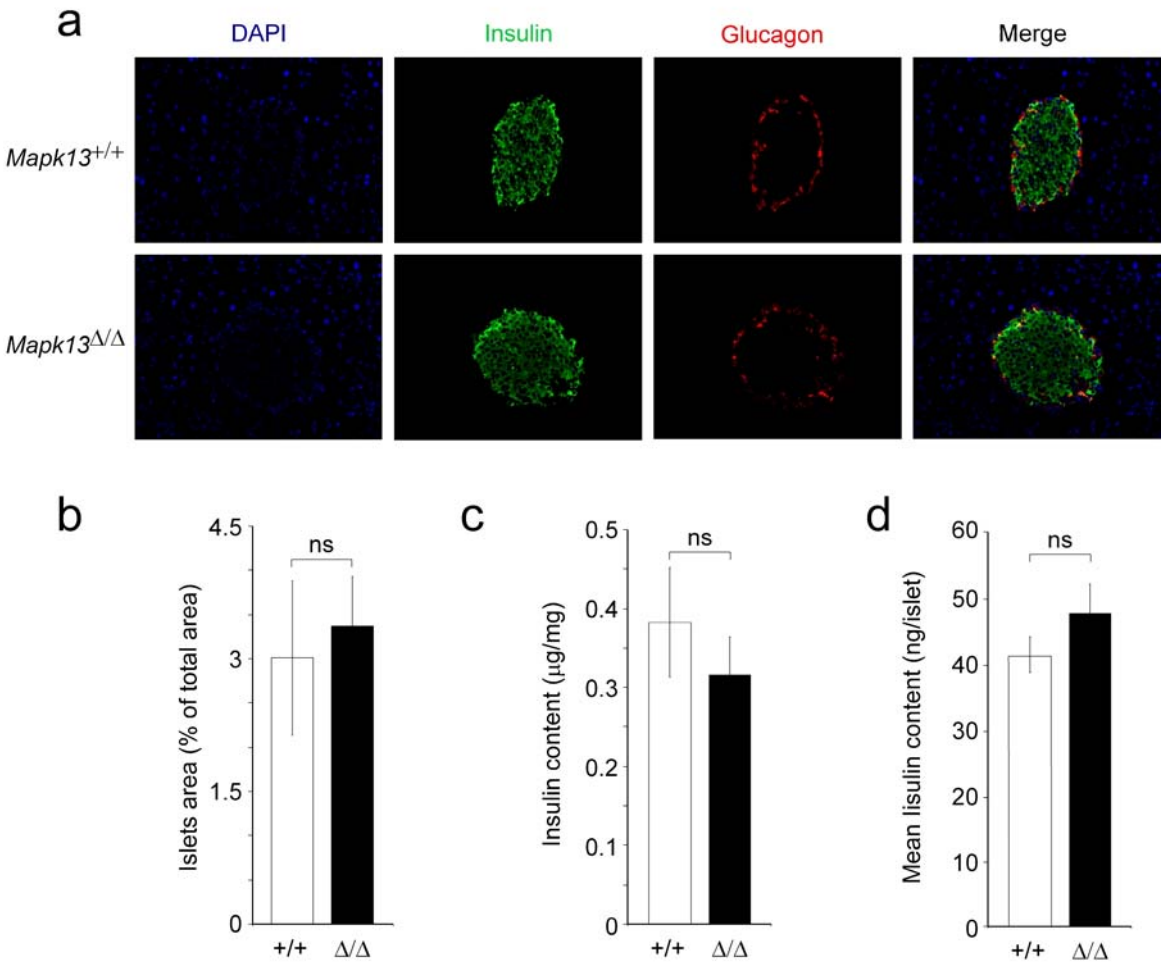

Figure 11 Unaltered islet architecture, islet area and insulin content in *Mapk13*$^{\Delta/\Delta}$ mice. (a) Insulin (middle left panel, green) and glucagon (middle right panel, red) immunolabeling revealed comparable islet characteristics in *Mapk13*$^{\Delta/\Delta}$ (lower panels) and *Mapk13*$^{+/+}$ (upper panel) mice. DAPI was used to stain nuclei (left panel). Merged pictures are provided in the right panels. (b) Quantitative histological assessment of total islet area in relation to total pancreatic area. No significant differences in *Mapk13*$^{\Delta/\Delta}$ (black bars, n=3) compared to *Mapk13*$^{+/+}$ (white bars, n=3) could be observed. (c) Total insulin extracted from pancreas measured by radio-immuno assays. No significant difference in total insulin content (µg/mg) was detected between *Mapk13*$^{\Delta/\Delta}$ (black bars, n=3) and *Mapk13*$^{+/+}$ mice (white bars, n=3). (d) The mean insulin content per isolated islet was also similar in *Mapk13*$^{\Delta/\Delta}$ (black bars, n=14) and *Mapk13*$^{+/+}$ mice (white bars, n=16).

This prompted us to address alterations in the insulin secretory pathway[205]. In the absence of *Mapk13*, insulin secretion from isolated islets was markedly enhanced during glucose stimulation (16.7 mM), but also in response to either 20 mM KCl or 100 µM

tolbutamide (Fig. 7d), indicating that p38δ acts downstream of glucose metabolism and K_{ATP} closure.

To determine whether the ablation of *Mapk13* affects the subsequent Ca^{2+} influx, we measured intracellular calcium concentrations ($[Ca^{2+}]_i$) in response to glucose and KCl in islets isolated from *Mapk13*$^{\Delta/\Delta}$ and *Mapk13*$^{+/+}$ mice. The average $[Ca^{2+}]_i$ was similar in the two groups of islets both under basal conditions (2.8 mM Glc) and after stimulation by either glucose (16.7 mM Glc) or KCl (20 mM in the presence of 2.8 mM Glc) (Fig. 12a and b). Likewise, whole-cell Ca^{2+} currents were comparable in single β cells of *Mapk13*$^{\Delta/\Delta}$ and *Mapk13*$^{+/+}$ littermates (Supplementary Fig. 12c and d). The fact that insulin secretion was enhanced in *Mapk13*$^{\Delta/\Delta}$ islets despite similar $[Ca^{2+}]_i$ levels suggests that p38δ acts downstream of the Ca^{2+} influx.

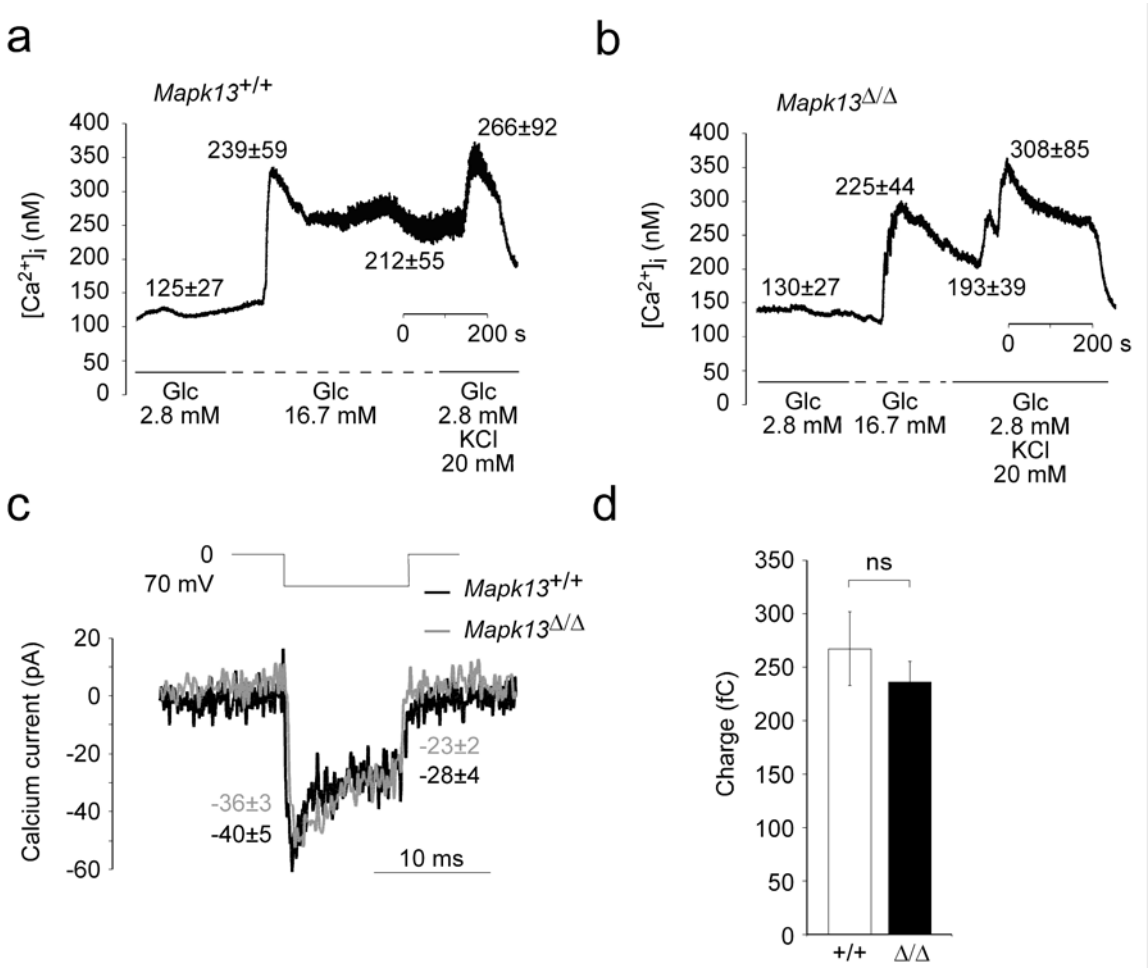

Figure 12 No effect of *Mapk13* deletion on $[Ca^{2+}]_i$ handling and voltage-gated Ca^{2+} currents. (a) and (b) Representative traces of intracellular calcium $[Ca^{2+}]_i$ in pancreatic islets of wild type (*Mapk13*$^{+/+}$) (n=16) (a) and *Mapk13* null (*Mapk13*$^{\Delta/\Delta}$) mice (n=12) (b) under basal conditions and after stimulation with 16.7 mM Glucose and 20 mM KCl. Average responses ±S.E are indicated for basal $[Ca^{2+}]_i$, peak glucose response, plateau glucose response, and the peak response to KCl. No significant difference could be observed between *Mapk13*$^{+/+}$ and *Mapk13*$^{\Delta/\Delta}$ islets. (c) Representative Ca^{2+} current in isolated pancreatic islets of *Mapk13*$^{+/+}$ (black solid line, n=20) and *Mapk13*$^{\Delta/\Delta}$ (grey solid line, n=17) mice evoked by a depolarisation from -70 mV to 0 mV. Currents are shown together with respective means ±S.E of the peak and sustained currents (measured at the end of the pulse). No significant difference was observed between *Mapk13*$^{+/+}$ and *Mapk13*$^{\Delta/\Delta}$ islets. (d) Summary of the average charge entry (derived from the area under the curve during the depolarisation) during the 10-ms pulse from -70 mV to 0 mV in *Mapk13*$^{+/+}$ (+/+) and *Mapk13*$^{\Delta/\Delta}$ (Δ/Δ) β cells. No significant difference was observed between *Mapk13*$^{+/+}$ and *Mapk13*$^{\Delta/\Delta}$ islets.

To address whether exocytosis is enhanced in *Mapk13*-deficient β cells, we performed high-resolution capacitance measurements of exocytosis on single β cells[206]. A train of 10 depolarization steps from -70 mV to 0 mV evoked larger responses in p38δ$^{\Delta/\Delta}$ than in control β cells, resulting in a 2.1 fold larger increase of membrane capacitance in *Mapk13* null compared to wild type cells (Fig. 13a and b). Capacitance of cells lacking *Mapk13* was also higher than that of wild type cells when exocytosis was elicited by dialysing the cell interior with a Ca^{2+}/EGTA buffer in order to obtain free Ca^{2+} concentrations of 1.5 μM, indicating that enhanced secretion occurs independently of Ca^{2+} influx (Fig. 13c). In these experiments, the rate of capacitance increase (Δc/Δt) was 50% higher in *Mapk13*$^{\Delta/\Delta}$ than in *Mapk13*$^{+/+}$ cells (Fig. 13d). These results indicate that in pancreatic β cells, p38δ acts as a negative regulator of insulin granule exocytosis, downstream of calcium signaling.

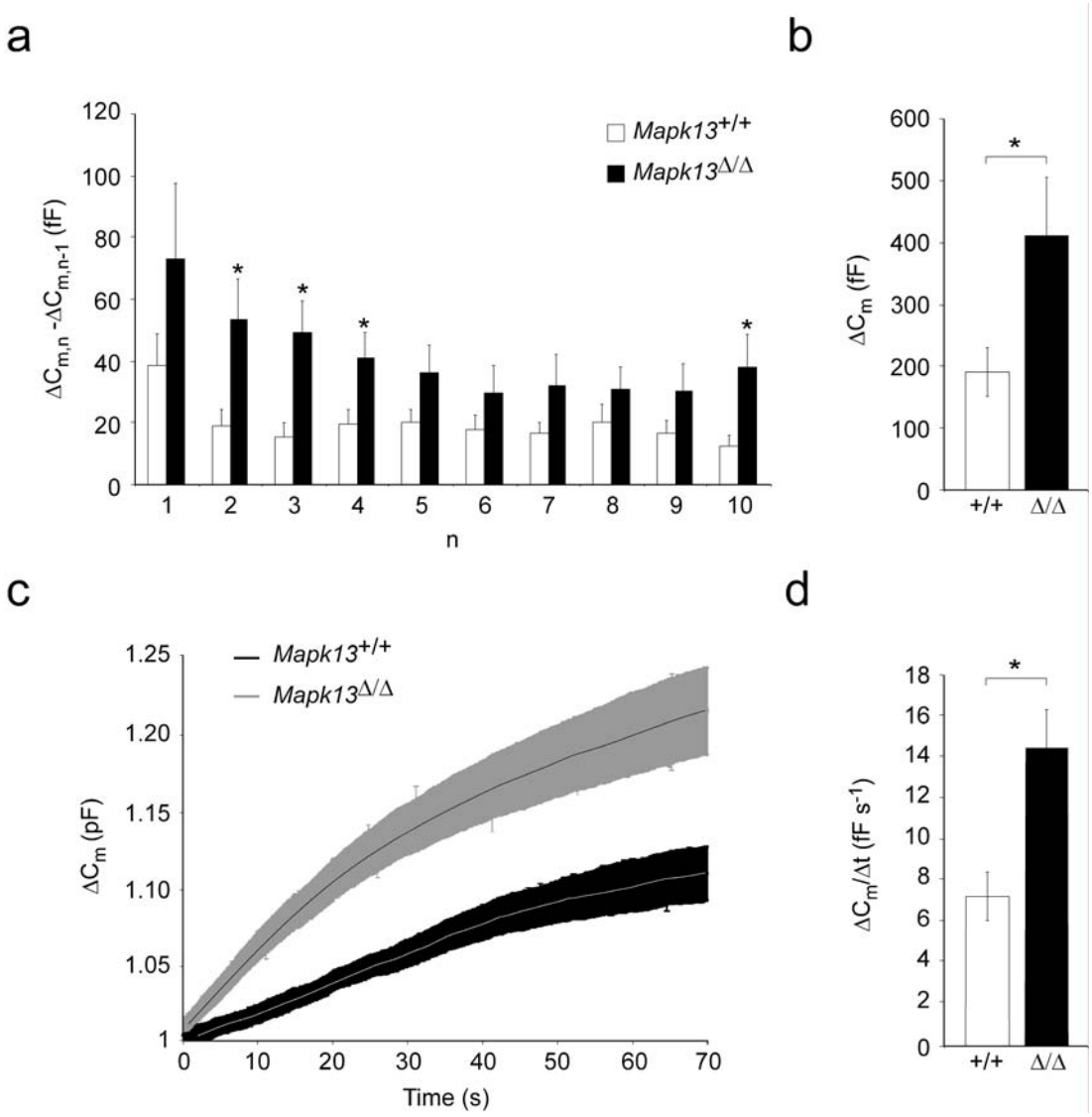

Figure 13 Calcium-independent enhancement of exocytosis in *Mapk13*-deficient pancreatic β cells. (a) A train of 10 successive 500 msec depolarizations from -70 to 0 mV increased capacitance (in femtofarad (fF)) in both *Mapk13*-deficient (p38δ$^{\Delta/\Delta}$) (black bars, n=15) and wild type *Mapk13*$^{+/+}$ (white bars, n=10) pancreatic β cells. The increase in capacitance evoked by the individual depolarizations was significantly larger (*p<0.05) in *Mapk13*$^{\Delta/\Delta}$ than in *Mapk13*$^{+/+}$ β cells, at depolarization steps 2, 3, 4 and 10. **(b)** Average cumulative increase of capacitance in *Mapk13*$^{\Delta/\Delta}$ (black bars) and *Mapk13*$^{+/+}$ (white bars) pancreatic β cells (*p<0.05). **(c)** Capacitance increase in picofarad (pF) elicited by intracellular application of 1.5 μM calcium via the recording electrode. Values have been normalized to the resting cell capacitance, which was 4.6±0.4 pF, n=8 and 4.6±0.2 pF, n=16, in wild type and mutant cells, respectively. Data are presented as the average normalised increase (central line) ±S.E (shaded areas). The grey curve corresponds to *Mapk13*$^{\Delta/\Delta}$ (n=16) while the black curve corresponds to *Mapk13*$^{+/+}$ (n=8). **(d)** Steady state average rate of capacitance change for *Mapk13*$^{+/+}$ (white bars, n=8) and *Mapk13*$^{\Delta/\Delta}$ (black bars, n=16) cells measured over a 60 sec period (*p<0.05).

To test whether p38δ activity affects the organization of organelles involved in exocytosis, MIN6 cells lacking *Mapk13* were compared to their corresponding controls using transmission electron microscopy. Inactivation of *Mapk13* in MIN6 cells resulted in formation of notedly more vesicles at the Golgi network compared to control cells (Fig. 14) All other organelles remained unaffected, including insulin-containing granules whose number and distribution were similar in *Mapk13* null and control cells, in agreement with the comparable insulin content of pancreas and islets in *Mapk13* null and control littermates (Fig. 11).

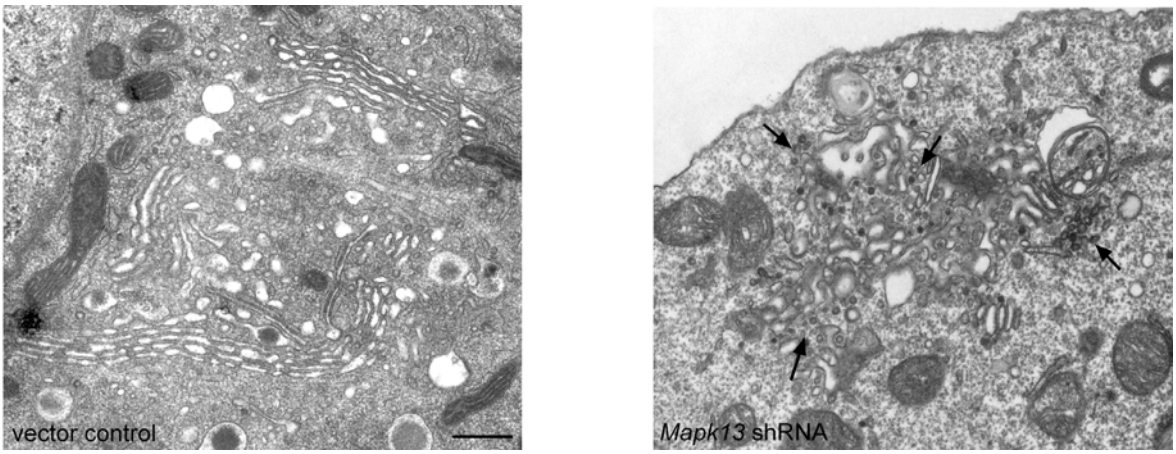

Figure 14 p38δ controls vesicle formation at the trans-Golgi network (TGN). Transmission electron microscopy showed that the Golgi apparatus of MIN6 cells stably expressing an empty vector (vector control) consisted of stacks of normal thin cisternae. In contrast, *Mapk13* shRNA-expressing MIN6 cells (*Mapk13* shRNA) displayed a fragmentation of the Golgi cisternae and increased vesicle formation at the TGN (arrows). Size bar 200 nm.

Budding of secretory vesicles from the Golgi complex occurs through membrane fission specifically at the trans-Golgi network (TGN), a key process regulating granule trafficking in secretory cells[207]. To assess whether these alterations mostly affected the TGN, we performed immunofluorescence using antibodies against proteins predominantly

localizing to the TGN. Inactivation of *Mapk13* led to a marked dispersion of giantin in MIN6 cells (Fig. 15a), as did the absence of *Mapk13* in cytospinned primary β cells (Fig. 16a).

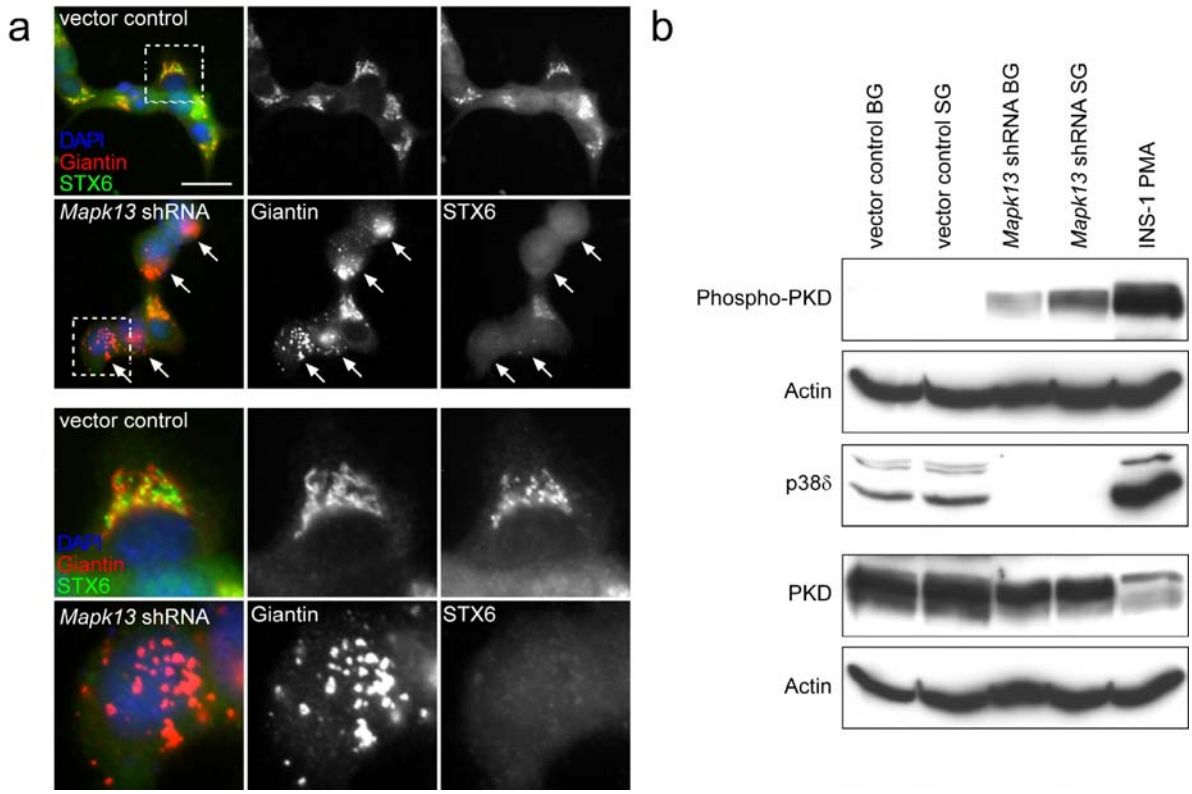

Figure 15 Enhanced membrane fission at the trans-Golgi network (TGN) and increased activity of PKD in *Mapk13*-deficient β cells. (a) Immunofluorescence using antibodies against Giantin (red) and syntaxin 6 (STX6, green) showed normal localization of these proteins at the TGN in MIN6 cells expressing the empty vector (vector control). In contrast, *Mapk13* shRNA expressing MIN6 cells displayed a larger, vesicular distribution of Giantin and lack of STX6 localization at the TGN (arrows). DNA was stained with DAPI (blue). Boxes in the left column outline the areas that have been magnified in the right panels. Size bar 10 μm. **(b)** Activity of protein kinase D (PKD) was determined by Western blotting using an antibody against activatory phosphorylation sites (serines 744 and 748). PKD activity was not detected in MIN6 control cells (vector control) but was evident in cells lacking *Mapk13* (*Mapk13* shRNA) (BG: basal glucose and SG: stimulatory glucose levels). Phorbol myristate acetate (PMA)-stimulated INS1 cells were used as a positive control (INS1 PMA). Immunoblotting with an antibody against actin was used to determine equal loading of phopho-PKD and total PKD blots.

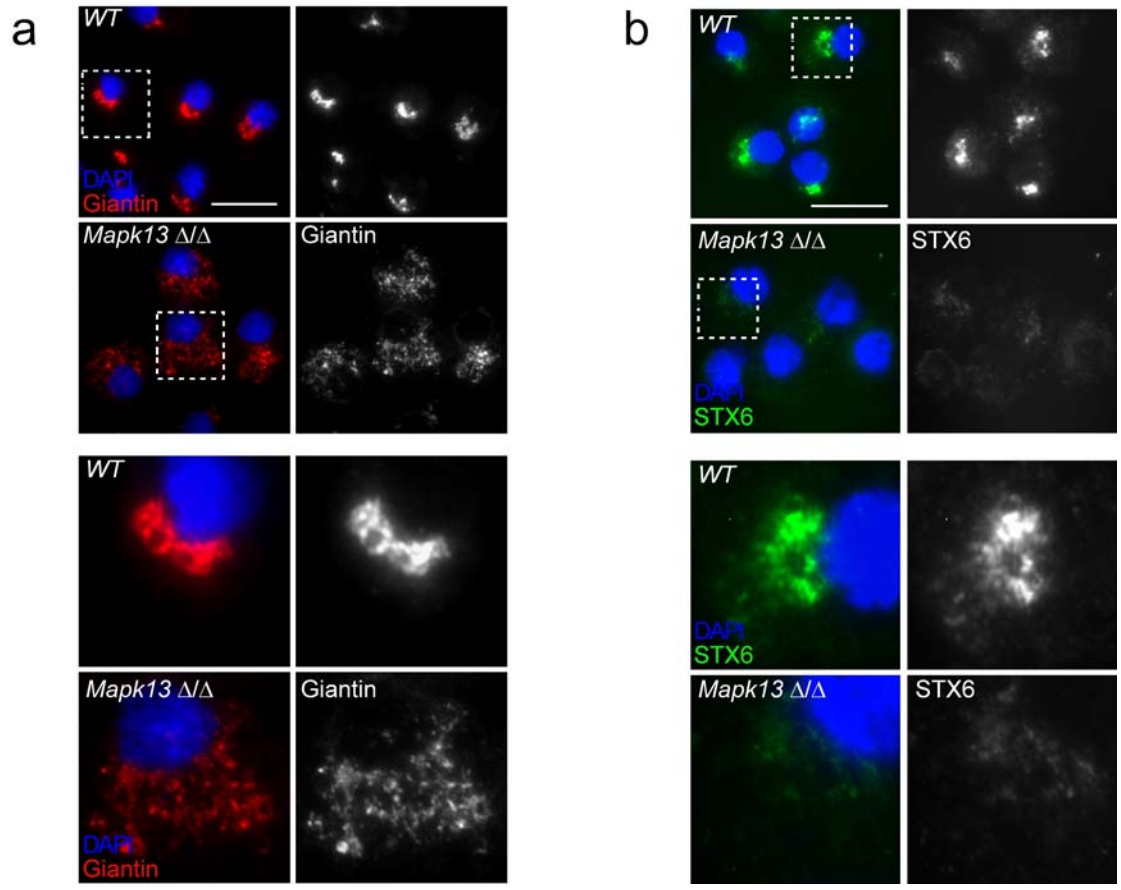

Figure S8 *Mapk13* deletion leads to dispersion of the trans-Golgi network proteins giantin and syntaxin 6 in primary β cells. (a) Immunofluorescence using antibodies against giantin (red) localized this protein in the TGN region of wild type *Mapk13*$^{+/+}$ (*WT*) primary β cells. The fluorescence labelling was markedly spread and showed a vesicular appearance in cytospinned primary β cells isolated from *Mapk13*$^{\Delta/\Delta}$ mice. Nuclear DNA was stained with DAPI (blue). Dashed boxes outline the areas that have been magnified in the panels below. Size bar 10 μm. **(b)** *Mapk13*-deficient (*Mapk13*$^{\Delta/\Delta}$) and wild type *Mapk13*$^{+/+}$ (*WT*) pancreatic islets were analyzed by immunofluorescence microscopy using an antibody against the TGN-protein syntaxin 6 (STX6, green). Nuclear DNA was stained with DAPI (blue). Dashed boxes outline the areas that have been magnified in the panels below. Note that *Mapk13*$^{\Delta/\Delta}$ cells show dispersion of STX6. Size bar 10 μm.

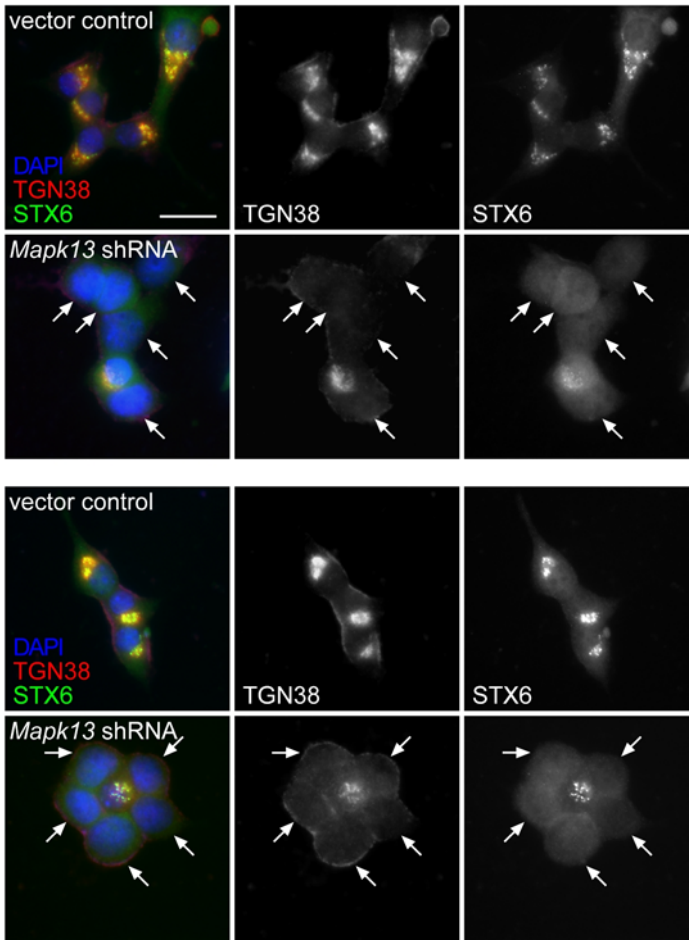

Figure 17 *Mapk13* deletion results in predominant localization of TGN38 to the plasma membrane. (A) MIN6 cells stably expressing shRNA against *Mapk13* (*Mapk13* shRNA) and cells expressing the empty vector (vector control) were cultured in the presence of basal (upper panel) or stimulatory glucose levels (lower panel) and analyzed by immunofluorescence microscopy using antibodies against TGN proteins TGN38 (red) and syntaxin 6 (STX6, green). DNA was stained with DAPI (blue). Note that *Mapk13* shRNA expressing cells show dislocalization of TGN38 and STX6 (arrows). TGN38 can be found on the plasma membrane in *Mapk13* shRNA-expressing cells (arrows).

Moreover, syntaxin-6 (STX6), a SNARE protein, which regulates membrane-trafficking from the TGN to the plasma membrane[208-210], failed to localize to the TGN and was dispersed throughout the cytoplasm of *Mapk13*-deficient MIN6 cells (Fig. 15a and Fig. 17) as well as *Mapk13*$^{\Delta/\Delta}$ β cells (Fig. 16b). TGN38, another protein primarily localized to the TGN, was found dispersed and translocated to the plasma membrane of MIN6 cells lacking

Mapk13, whereas it predominantly localized to the TGN of control cells (Fig. 17). Conversely, the expression of the constitutively active p38δ in INS1 cells led to enhanced accumulation of STX6 and TGN38 proteins in the TGN (Fig. 18). Moreover, giantin immunofluorescence showed that expression of the constitutively active p38δ caused tubular protrusions from the Golgi apparatus (Fig. 19). Overall, these data support a specific role of p38δ in the organization of the TGN of pancreatic β cells.

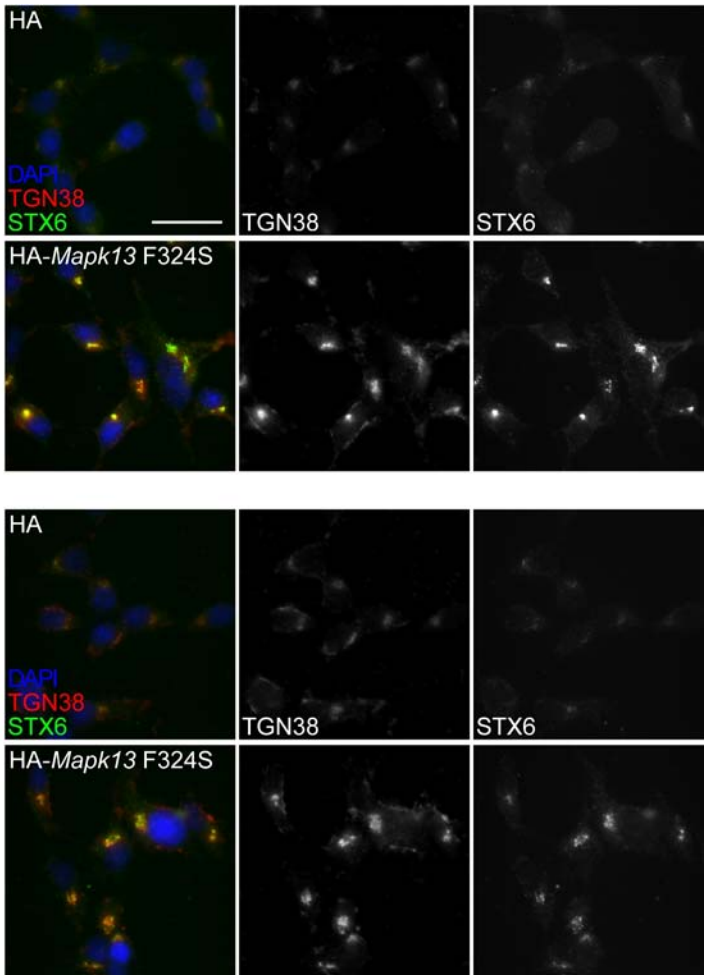

Figure 18 Ectopic expression of constitutively active p38δ results in enlargment of the trans-Golgi network. INS1 cells stably expressing hemagglutinin (HA) tag or HA-tagged constitutively active p38δ (HA-*Mapk13*^{F324S}) were cultured in the presence of basal (upper panel) or stimulatory glucose levels (lower panel) and immunostained with antibodies against TGN proteins TGN38 (red) and syntaxin 6 (STX6, green). Nuclear

DNA was stained with DAPI (blue). Expression of p38δ^{F324S} led to enhanced and focused staining of TGN38 and syntaxin 6 indicating enlargment of the TGN. Size bar 10 μm.

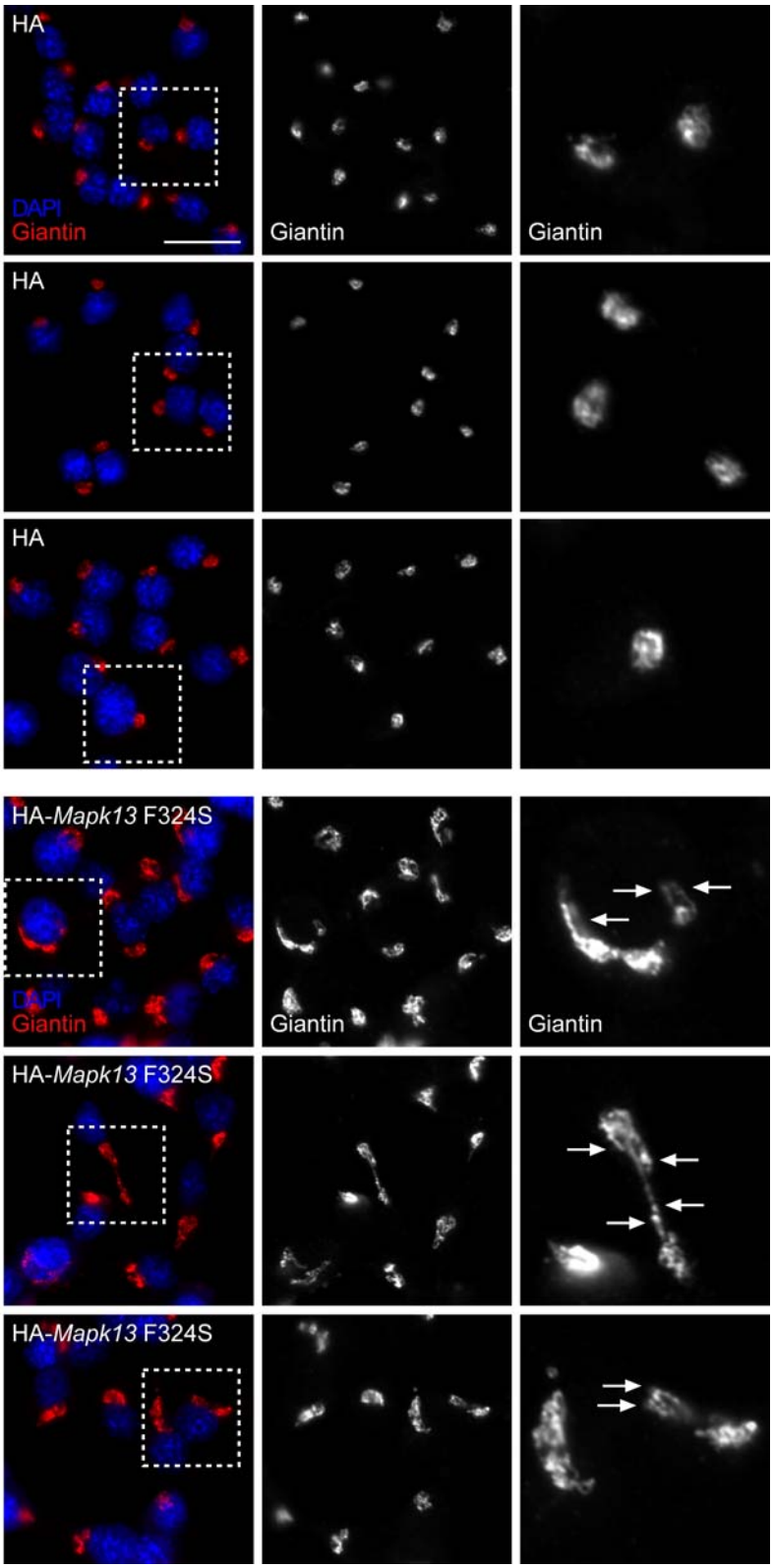

Figure 19 Ectopic expression of constitutively active p38δ leads to formation of tubular protrusions from the Golgi complex. INS1 cells stably expressing hemagglutinin (HA) tag or HA-tagged constitutively active p38δ (HA-*Mapk13*^{F324S}) were cultured in the presence of stimulatory glucose levels and immunostained with antibodies against giantin (red). DNA was stained with DAPI (blue). Boxes in the left column outline the areas that were magnified in the panels on the right side. Expression of p38δ^{F324S} led to formation of tubular protrosions from the Golgi complex (arrows). Size bar 10 μm.

The protein kinase D (PKD) has evolved as a major player in membrane fission at the TGN, which is required to generate transport carriers en route to the plasma membrane[211, 212]. Inhibition of PKD activity blocks secretion by preventing membrane fission and formation of tubular Golgi protrusions[213, 214]. In contrast, PKD overactivation enhances membrane fission and increases secretion[213, 215]. Therefore, we compared phosphorylation of the activatory sites of PKD in MIN6 cells lacking *Mapk13* and in control cells in response to basal and stimulatory glucose levels using Western blotting. No detectable PKD activity could be seen in control MIN6 cells, neither under basal nor stimulatory glucose concentrations (Fig. 15b). In contrast, a marked activation of PKD was observed in MIN6 cells lacking *Mapk13*, which was further enhanced when these cells were stimulated by glucose (Fig. 15b). We then tested whether inhibition of PKD activity in *Mapk13*$^{\Delta/\Delta}$ primary β cells reconstituted TGN integrity and insulin secretion. Generation of diacylglycerol (DAG) by phosphatidyl-inositol-specific phospholipases C (PI-PLCs) at the TGN activates PKD and recruits it to this organelle to allow efficient membrane fission. We used the specific PI-PLC inhibitor U73122, which has been shown to inhibit PKD activity and transport from the TGN to the plasma membrane[24] and Gö6976, a potent inhibitor of PKD and conventional protein kinases C (PKCs)[216]. We observed that both U73122 and Gö6976 resulted in relocalization of Giantin to the TGN in *Mapk13*$^{\Delta/\Delta}$ primary β cells, whereas none of the compounds altered the normal localization of Giantin in *Mapk13*$^{+/+}$ cells (Fig. 20a). Furthermore, pre-treatment with U73122 or Gö6976

resulted in similar amounts of insulin secretion in response to 16.7 mM glucose (Fig. 20b). Most importantly, peritoneal injections of U73122 decreased the glucose tolerance of *Mapk13*$^{\Delta/\Delta}$ mice to levels observed in DMSO-treated *Mapk13*$^{+/+}$ controls, without modifying the glucose tolerance of the latter animals (Fig. 20c).

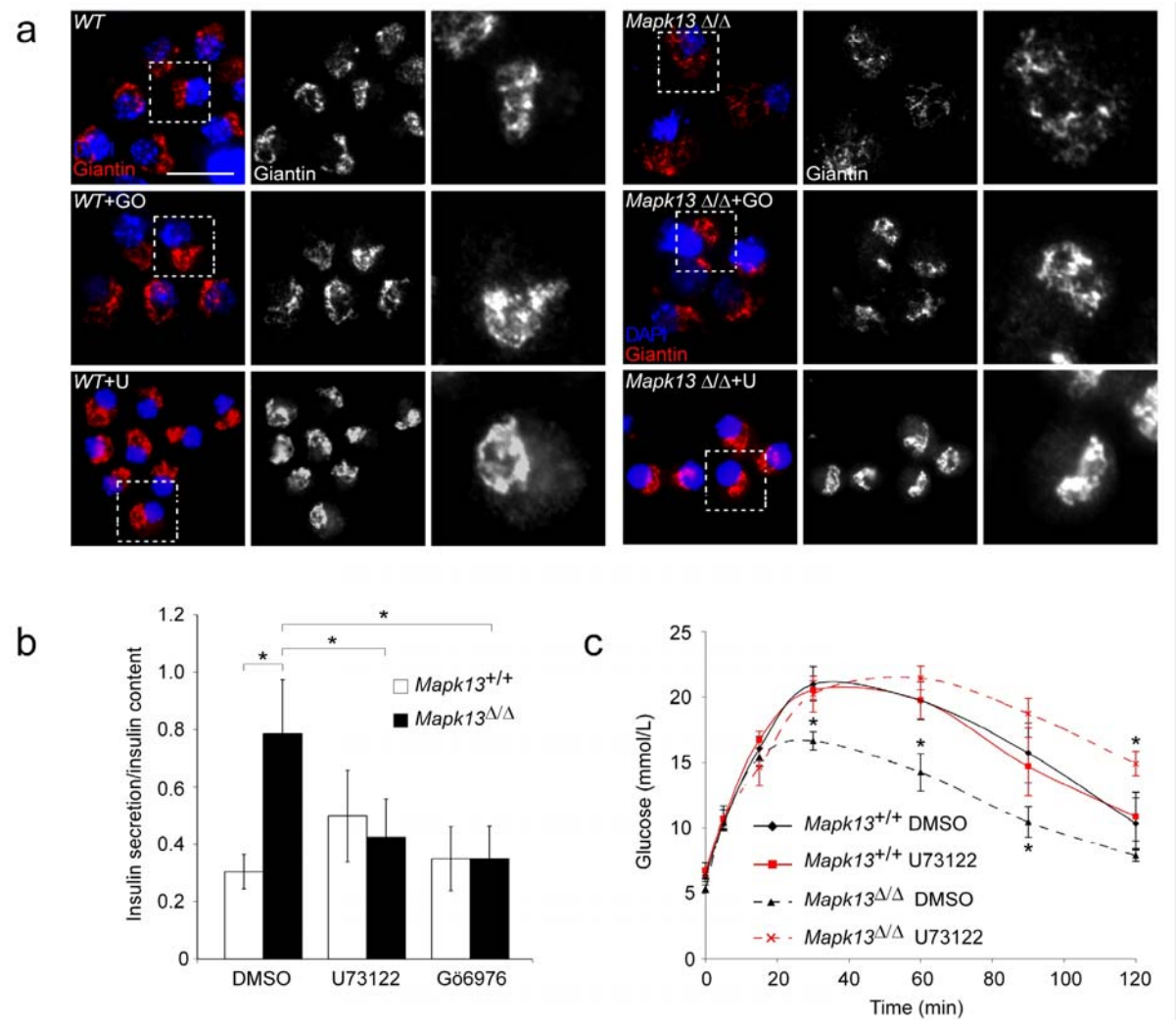

Figure 20 PKD inhibition reconstitutes insulin secretion in primary β cells and restores glucose tolerance in *Mapk13* null mice. (a) Exposure to Gö6976 and U73122 resulted in the relocalization of Giantin to the TGN in *Mapk13*$^{\Delta/\Delta}$ primary β cells but did not affect its localization in *Mapk13*$^{+/+}$ (WT) cells. DNA was stained with DAPI (blue). Boxes in the left column outline the areas that have been magnified in the right panels. Size bar 10 μm. (b) Glucose (16.7 mM)-stimulated insulin secretion was significantly higher from *Mapk13*$^{\Delta/\Delta}$ islets (black bar, n=5) (*$p<0.05$) than from *Mapk13*$^{+/+}$ islets (white bar, n=5) exposed to DMSO. Both, U73122 and Gö6976

reduced insulin secretion of *Mapk13*$^{\Delta/\Delta}$ islets (black bars, n=5) (*p<0.05) to the levels observed in *Mapk13*$^{+/+}$ islets (white bars, n=5). (c) Glucose (2 g/kg b.w.) was injected intraperitoneally after a 16 h fasting, and plasma glucose levels were measured at indicated time points. DMSO-treated *Mapk13*$^{\Delta/\Delta}$ mice (triangles and dashed black line, n=6) showed an improved tolerance to glucose compared to *Mapk13*$^{+/+}$ controls (*p<0.05). This difference was abolished by treatment with U73122 (crosses and dashed red line, n=6) that reduced the glucose tolerance of p38δ$^{\Delta/\Delta}$ mice to the levels observed in *Mapk13*$^{+/+}$ (U73122: squares and solid red line, n=5 and DMSO: diamonds and solid black line, n=5).

These experiments demonstrate that enhanced membrane fission at the TGN is fully reversible in *Mapk13*-deficient β cells and thus might represent a critical step enhancing insulin secretion and glucose tolerance in *Mapk13*$^{\Delta/\Delta}$ mice. Pharmacological inhibition of p38δ might represent an innovative and attractive therapeutic approach to enhance insulin secretion in diabetic patients.

7. Methods

Generation of mice. The *Mapk13* floxed mice were generated at Lexicon Pharmaceuticals (The Woodlands, TX). The *Mapk13* targeting vector was derived using the Lambda KOS system[217]. The Lambda KOS phage library, arrayed into 96 superpools, was screened by PCR using primers *Mapk13*-2 [5'-CCTCGCAGGACCGCCACCAC-3'] and *Mapk13*-4 [5'-GGGCGCCAGGTAGGTCTTGG-3']. The PCR-positive phage superpools were plated and screened by filter hybridization using the 114 bp amplicon derived from primers *Mapk13*-2 and *Mapk13*-4 as a probe. Three pKOS genomic clones, pKOS-50, pKOS-63, and pKOS-70 were isolated from the library screen and confirmed by sequence and restriction analysis. Gene-specific arms (5'-GAACGTACCTGGGCGAGGCGGCAGGT-3') and (5'-AGTTACAGCCTTGGAGAACCAGATTCG-3') were appended by PCR to a yeast selection cassette containing the URA3 marker. The yeast selection cassette and pKOS-50 were co-transformed into yeast, and clones that had undergone homologous recombination to replace a 346 bp region containing exon 1 with the yeast selection cassette were isolated. This 346 bp fragment was independently amplified by PCR and cloned into the intermediate vector pLF-Neo introducing flanking LoxP sites and a Neo selection cassette (*Mapk13*-pLFNeo). The yeast cassette was subsequently replaced with the *Mapk13*-pLFNeo selection cassette to complete the conditional p38δ targeting vector that has exon 1 flanked by LoxP sites. The Not I linearized targeting vector was electroporated into 129/SvEv^{Brd} (Lex-1) ES cells. G418/FIAU resistant ES cell clones were isolated, and correctly targeted clones were identified and confirmed by Southern analysis using a 413 bp 5' external probe (57/58), generated by PCR using primers p38δ-57 [5'-GGCACATGGCAAGCACGTATG-3'] and *Mapk13*-58 [5'- CTAATTCTGAGTATAGATCTTTGC-3'], and a 313 bp 3' internal probe (60/59), amplified by PCR using primers *Mapk13*-60 [5'-CGTGGGTGCTAAGGGTTGAAC-

3’] and *Mapk13*-59 [5’- GGTAGCTGGAAGGCAGGAGTG-3’]. Southern analysis using probe 57/58 detected a 5.5 Kb wild type band and 3.2 Kb mutant band in Eco RI + Sfi I digested genomic DNA while probe 60/59 detected a 15.5 Kb wild type band and 13.8 Kb mutant band in Xho I + Sfi I digested genomic DNA. Targeted ES cell clones were microinjected into C57BL/6 (albino) blastocysts to generate chimeric animals which were bred to C57BL/6 (albino) females, and the resulting heterozygous offspring were bred with a protamine-Cre recombinase transgenic line[203] to delete exon 1 (Fig. 6). Mice heterozygous for exon 1 deletion were backcrossed four times to the C57BL/6 background and intercrossed to generate homozygous knockout mice. Genotyping was performed by PCR using following primers: Delta-1 (5’-GAGCTACCCAAGACCTACCTG-3’, PGK-162r (5’-GGATGTGGAATGTGTGCGAGG-3’) and BI5-12 (5’-GCTCAGCTTCTTGATGGCCAC-3’) yielding a 744 bp band corresponding to the floxed allele and a 560 bp band corresponding to the wild type allele; Delta-2 (5’-ACGTACCTGGGCGAGGCGGCA-3’) and BI5-12 (5’-GCTCAGCTTCTTGATGGCCAC-3’) yielding a 799 bp wild type band and a 555 bp band corresponding to the deleted *Mapk13* allele.

Animal experiments. For the glucose tolerance test, 8-week-old male mice were fasted for 16 hr and then injected intraperitoneally with glucose (2 g/kg b.w.). Glucose was measured in blood from the tail vein at indicated time points using Accu-Chek Aviva system from Roche. In experiments with the PI-PLC inhibitor U73122 (Sigma), mice were injected with U73122 (2.5 mg/kg b.w.) in DMSO intraperitoneally or DMSO only 1h prior experiment. Insulin tolerance tests were performed on 8-week-old ad libitum fed male mice injected intraperitonealy with insulin (1U/kg b.w.). Plasma insulin levels during glucose tolerance test were measured using an insulin RIA kit (Linco).

Western blotting. For Western blotting the following antibodies were used: rabbit-anti-p38δ (Santa Cruz Biotechnology) and sheep-anti-p38δ (generous gift of Dr. Ana Cuenda), anti-phospho-PKD (Cell Signaling Technology), anti-PKD, anti-HIS, anti-hemagglutinin (HA), anti-tubulin and anti-Actin (all from Sigma).

Real-time RT-PCR. RNA was extracted using TRIzol (Invitrogen). cDNA was synthesized using the Ready-To-Go You-Prime First-Strand beads (Amersham). Following primers were used: *Mapk13* forward: 5'-ATGAGCCTCACTCGGAAAAGG-3', *Mapk13* reverse: 5'-GCATGTGCTTCAAG AGCAGAA-3', *18S* forward: 5'-GTTCCGACCATAAACGATGCC-3' *18S* reverse: 5'-TGGTGGTGCCCTTCCGTCAAT-3'.

***In vitro* kinase assay.** 293 cells were transfected with a plasmid carrying HA-tagged wt or a constitutive active *Mapk13* isoforms (D176A and F324S), lysed and incubated with anti-HA antibody (Sigma) bound to agarose beads for 2h at 4°C. Immunoprecipitates were washed an incubated for 30 min at 30°C in kinase buffer containing 20 μM ATP, 5 μCi [γ-^{32}P] ATP and 2 mg of recombinant his-tagged ATF-2 (Santa Cruz Biotechnology).

Generation of stable cell lines. INS-1 cells were generated by transfection with the pcDNA3.1-HA- *Mapk13*, pcDNA3.1-HA- *Mapk13*^{F324S} expression plasmid[218] or empty vector using Fugene reagent (Roche) and selected with 500 μg/ml neomycin (Sigma). Knockdown of *Mapk13* in MIN6 was obtained by lentivirus-mediated transduction of short hairpin against *Mapk13* RNA (5'-GATCCCCGATGCTGGAGCTGGATGTGTTCAAGAGACACATCCA GCTCCAGCATCTTTTTA-3'; 5'-AGCTTAAAAAGATGCTGGAGCTGGATGTGTCTCT TGAACACATCCAGCTCCAGCATCGGG-3') and selection with puromycin (4μg/ml).

Islets isolation and insulin secretion. Islets were isolated by collagenase perfusion (1.9 U/ml) of pancreas and subsequent digestion for 16 min at 37ºC. Islets were handpicked and transferred to RPMI1640 media containing 5mM glucose, and maintained for 3h prior to the experiments or dispersed in calcium-free solution and then cultured as above. For static incubations islets were subsequently pre-incubated for 60 min at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 0.05% BSA, and 2.8 mM glucose. Following pre-incubation, the supernatant was discarded and the islets were incubated in buffer solution containing 2.8 or 16.7 mM glucose for 60 min at 37°C. For the rescue experiments islets were incubated as described above but in presence of 10 μM of U73122 or 1 μM Gö6976 or DMSO. The islets were resuspended in acid ethanol and frozen for insulin content measurements. Insulin secretion data were expressed as percentage of pellet. Insulin secretion from INS1 and MIN6 cells was performed as described above. Total insulin from pancreas was isolated using acid-ethanol extraction. Insulin content was normalized to the total weight of the pancreas.

Electrophysiology. Whole cell currents and exocytosis were recorded and analysed using EPC-9 patch-clamp amplifiers and the software pulse+pulsefit (Heka Electronic). Exocytosis, expressed as an increase in capacitance was elicited by either a train of 10 500 ms depolarisations delivered at 1 Hz, from -70 mV to 10 mV or by infusion of a Ca^{2+}/EGTA-buffer with an intracellular free $[Ca^{2+}]_i$ of 1.5 μM. The electrodes were pulled from borosilicate glass and had a resistance of 4 to 7. When filled with the electrode solution, the access resistance was <15 MΩ and seal resistance > 1 GΩ. β-cells were identified as previously described[219]. In the Ca^{2+} dialysis experiments, the intracellular solution consisted of (in mM) 125 CsCl, 10NaCl, 1 $MgCl_2$, 10 EGTA, 9 $CaCl_2$, 3 Mg-ATP, 0.1 cAMP, 5 Hepes (pH 7.15). When exocytosis was triggered by depolarization, the intracellular solution contained (in mM) 125 glutamate, 10 CsCl, 10 NaCl, 1 $MgCl_2$, 5 Hepes, 0.05 EGTA, 0.1

cAMP and 3 MgATP (pH 7.15). For all experiments, cells were superfused with a solution containing 118 NaCl, 20 TEA-Cl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 25 Hepes, 5 glucose (pH 7.4).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was assessed in freshly isolated islets using a dual wavelength PTI system (PTI, Monmouth, NJ) fitted on an inverted microscope. Islets were loaded with 3 μM fura-2AM in the presence of 0.007% w/v pluronic acid (Invitrogen) for 25 min at 37ºC. Islets were held in the chamber using a fire polished borosilicate glass pipette. In solution composed of (in mM) 140 NaCl, 3.6 KCl, 2 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgSO_4$, 5 Hepes (pH 7.4 with NaOH), 2.6 $CaCl_2$ preheated with temperature controller (TC 324B, Warner instrument corporation) at 37ºC. Glucose and KCl were included in the extracellular medium as indicated. When KCl was elevated, NaCl was correspondingly reduced. The fluorofore was excited at 350 and 380 nm. Emitted light was collected at 510 nm. Ratiometric measurements were done at 25 Hz and calibrated as described[220]. R_{max} was measured after addition of ionomycin (conc: ~1 μM) in the presence of 10 mM $CaCl_2$ at the end of the experiment. R_{min} was established by replacing the extracellular solution with 0 mM $CaCl_2$. Background was obtained after quenching fura-2 fluorescence with 1 mM $MnCl_2$.

Immunofluorescence. Immunofluorescence for insulin (Linco) and glucagon (Linco) was performed on paraformaldehyde-fixed pancreatic sections. Relative islet area was measured as percentage of insulin-positive staining sections. Ten different sections per mouse were used. Immunofluorescence for syntaxin 6 (BD Bioscience), Giantin (Covance) and TGN38 (Abcam), were performed on methanol-acetone fixed INS1 and MIN6 cells and primary β-cells. Islets were dispersed in single cells as described above, incubated in RPMI 1640 medium containing 5 mM glucose for 2h in the presence or abscence of the PI-PLC inhibitor

U73122 (10 μM), the PKC inhibitor Gö6976 (1 μM) or DMSO and then spun onto poly-lysine-coated slides (700 G for 5 min).

Electron microscopy. For transmission electron microscopy, MIN6 cells have been directly fixed on culture dishes in 3% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4), embedded in Araldite (Serva, Heidelberg, Germany), cut, and contrasted with uranyl acetate and lead citrate. Photographs were taken with a Philips CM 10 or EM 300 electron microscope.

Statistical analysis. Data are presented as mean values ± SE. Statistical significance was calculated using an ANOVA with post-hoc Tukey's test and Student unpaired *t* test, respectively. Significance was accepted at the level of $p<0.05$.

7. Discussion

Three major groups of the mitogen activated protein kinase (MAPK) family have been identified in mammals: extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs[221, 222]. An extensive research activity defined particular functions for MAPK in immunity[223]. More recently, JNK has been discovered to be a critical regulator of obesity and insulin resistance[4].

Similar to the JNK pathway, the closely related p38 signaling cascade appears to mediate various molecular events in response to inflammatory and metabolic stimuli in different cell types and organs.

Four isoforms of p38 MAPK encoded by four different genes have been identified: p38α[126], p38β[127], p38γ [128] and p38δ[129]. Activation of p38 occurs via dual phosphorylation of the conserved TGY motifs by the MAPK kinases, MKK6 and MKK3[117]. Over the last decade, various substrates of p38 have been identified. Moreover, innumerable p38 expression and activation studies have been conducted in animal disease models, in patients and *in vitro* models. Although some of the available data seem to be controversial, these studies suggest that p38 represents a key regulator of many basic biological processes such as proliferation, differentiation, stress response, development, tumorgenesis, inflammation and apoptosis and thus could be involved in several diseases[9, 224]. However, the requirement of p38 in all of these processes *in vivo* and even *in vitro* and the specific roles of p38 isoforms are largely unknown.

In our study, we showed that unlike other isoforms of the p38 family, the p38δ expression pattern is largely restricted to pancreatic islets and exocrine pancreas. Moreover, expression of p38δ could not be detected in other organs involve in regulation of glucose homeostasis. This specific pattern of expression prompted us to investigate p38δ function in

pancreatic β cells and regulation of glucose homeostasis. To address these questions, we generated p38δ knockout mice. Consistently with previous reports, these mice were viable, fertile and did not show any obvious phenotype[184]. However, p38δ-deficient mice showed improved glucose tolerance due to enhanced insulin secretion caused by increased insulin granule exocytosis from pancreatic β cells. p38δ seems to specifically regulate glucose homeostasis by regulation of insulin release since the levels of glucagon, another key hormone involved in regulation of serum glucose levels, which is specifically secreted by α cells in the pancreatic islet, are not affected by deletion of this kinase. Since enhanced activity of pancreatic β cells has a suppressive effect on glucagon secretion[225], we did not fully exclude that α cells lacking p38δ also show enhanced secretion and that enhanced activity of β cells observed in these mice would mask this effect. For this purpose, α cells should be specifically isolated and tested for their secretion capacity. Alternatively, this might indicate inhibitory mechanisms mediated by p38δ are present or necessary only in certain secretory cell. Our experiments also demonstrate that deletion of p38δ does not affect peripheral insulin sensitivity, which is in line with the lack of expression of p38δ in peripheral insulin-sensitizing organs. We can also not exclude whether p38δ mediates the hypothalamic regulation of glucose levels[226]. In fact, a recent study indicates that a subset of hypothalamic pro-opiomelanocortin (POMC) neurons regulate peripheral glucose disposal in response to elevated glucose levels. Moreover, POMC neurons are sensing glucose utilizing similar molecular mechanisms as reported for pancreatic β cell[227], therefore a possible involvement of p38δ in hypothalamic regulation of glucose homeostasis should be investigated in future.

Since p38δ not only is expressed in pancreatic β cells but also is highly abundant in neutrophils (Ittner A. et al. unpublished data), we also studied the function of this kinase in neutrophils, which, as the pancreatic β cells, represent specialized granulated secretory cells. Intriguingly, neutrophils lacking p38δ likewise show hypersecretory features (enhanced

degranulation), which affects their recruitment to inflammatory sites and overall migratory capacity, highlighting the importance of p38δ in neutrophil-dependent innate immunity (Ittner A. et al. unpublished data).

Most intriguingly, pancreatic β cells (primary cells as well as cell lines) lacking p38δ showed a very particular and consistent phenotype in the trans-Golgi network (TGN). By immunofluorescence and transmission electron microscopy, we have found that the TGN formed markedly more vesicles and that several TGN-resident proteins are constitutively dispersed, a phenomenon, which typically occurs when membrane fission at the TGN is enhanced to trigger more efficient vesicle transport from the TGN to the plasma membrane. Most important in this context, these mechanisms have been shown to be crucial to enhance regulated secretion of cargo proteins (i.e. neurotensin secretion from neuroendocrine cells)[212, 228, 229] and therefore they might explain the observed enhanced insulin secretion in the absence of p38δ. To further corroborate this hypothesis, we considered recent exciting studies (published in *Cell*, *Science*, *Journal of Cell Biology* and *Nature Cell Biology*) that demonstrated that membrane fission at the TGN is in fact tightly regulated at the molecular level. It is now established that protein kinase D (PKD) is specifically required to promote membrane fission at the TGN[212-215, 230-232]. Consistently, we see PKD constitutively activated in β cells lacking p38δ. Most importantly, we were able to demonstrate that inactivation of PKD, using inhibitors, fully restores enhanced membrane fission at the TGN in p38δ-deficient cells and reconstitutes normal insulin secretion in islets lacking p38δ as well as glucose tolerance in p38δ null mice. Overall, we present strong evidence that p38δ is an important negative regulator of insulin secretion that links PKD-dependent TGN membrane fission in pancreatic β cells to insulin secretion and glucose homeostasis.

PKD itself is activated by specific upstream signaling events: Upon stimulation of cells, most probably mediated by a G protein coupled receptor (GPCR), the GTP-binding

protein β1γ2 subunit translocates to the membrane of the TGN and activates phosphatidyl-inosytol phopholipase C β3 (PI-PLCβ3), which results in generation of a second messenger molecule, diacylglycerol (DAG). DAG is required to activate protein kinase C η (PKC η) which in turn recruits PKD to the membrane of TGN. Once recruited to the TGN, PKC η phosphorylates PKD at the activatory sites[215] (Fig. 21). We have shown that inhibition of PKD activity using a phosphatidyl-inosytol phopholipases C (PI-PLCs) specific inhibitor U73122 or Gö6976, a potent inhibitor of PKD and conventional PKC isoforms, restores membrane fission from TGN and insulin secretion in p38δ-deficient β cells to the levels observed in wild type cells. Moreover, inhibition of PKD *in vivo* in p38δ knockout mice using U73122 inhibitor decreased glucose tolerance to levels observed in wild type animals, whereas the same substance had no effect on glucose tolerance in wild type mice. Taken together, these data suggest that p38δ-dependent suppression of membrane fission at the TGN and insulin secretion from pancreatic β-cells is achieved by suppression of PKD activity.

However, the exact molecular mechanisms of p38δ-dependent suppression of PKD activation is unclear. The fact that inhibition of PI-PLC in p38δ-deficient cells is sufficient to rescue the phenotype, might suggest that p38δ rather acts upstream of PI-PLC, for example by desensitizing the GPCR to its ligand, by blocking the action of the Gβγ subunit or by directly inhibiting PI-PLC action. Still, it is also possible that p38δ directly inhibits PKC η or PKD. Eventually, full activation of PKD or PKC η might require both activation by DAG but also abolition of a direct p38δ-dependent inhibitory effect on PKC η and or PKD (Fig. 1). The second hypothesis is supported by the fact that activation of PKD is notedly more pronounced in the presence of stimulatory glucose levels when the cellular content of DAG increases only in the absence of p38δ. In wild type cells, despite the fact that DAG generation is enhanced in pancreatic β cells upon glucose stimulation[1] PKD activity and thus membrane fission is kept at very low levels, most likely by p38δ. This would also imply

that p38δ sets a threshold to control membrane fission at the TGN. In our laboratory, we recently obtained strong biochemical data, which fully support a direct regulation of PKD activity by p38δ (Formentini I et al. unpublished data). p38δ physically interacts with PKD and directly phosphorylates it at specific serine residues (Ser 397 and Ser 401), both of which represent conserved p38 MAPK phosphorylation sites. Future experiments will be conducted to analyze these phospho-sites functionally regarding their potential inhibitory nature.

Another key question remains: What are the upstream factors/pathways regulating p38δ activity? And along the same line, what are the precise physiologic conditions in which p38δ activity has to be diminished/enhanced to allow/prevent PKD to exert membrane fission at the TGN? Although previous studies established several pro-inflammatory agents, which activate p38δ[129], other physiological stimuli, which may lead to activation or deactivation of p38δ are not yet identified. In our study, we also showeded that the kinase activity of p38δ is sufficient to inhibit insulin secretion and vesicle fission from the TGN, since ectopic expression of constitutive active but not the wild-type form of p38δ suppressed the release of insulin and fission of vesicles from TGN. Potential activators of p38δ should decrease the release of insulin. Several agents and physiologic conditions may serve as potential factors activating p38δ. Among others, low glucose levels and the release of sympathetic neurotransmitters such as norepinephrine or catecholamines (i.e. under fasting conditions) should be considered. In fact, enhanced insulin secretion in p38δ knockout mice can be predominantly seen after fasting them (data not shown), while ad libitum fed mice show almost no differences in insulin release. On the other hand, it is possible that in pancreatic β cells, a certain pool of p38δ is constitutively active and stimulation with insulinotropic agents such high glucose, FFA, GLP-1 or acetylcholine could result in p38δ inactivation and more efficient fission of secretory granules at the TGN. Interestingly enough, many of the factors mentioned above act through GPCRs, the latter of which, although unidentified yet, has been

proposed to regulate the activity of PKD. In particular, it should be mentioned here that acetylcholine, binding mainly to M3 muscarinic receptors, activates PI-PLC to generate DAG and to enhance insulin secretion[233]. Experiments with these agents and conditions in the absence or presence of p38δ/PKD have to be conducted in the near future.

Several lines of evidence suggest that activation of multiple pathways, which initially enhance insulin secretion, in the long term (i.e during the state of peripheral insulin resistance) might lead to pancreatic β cell failure[15, 83, 96, 99]. In our study, we so far have used mostly 8 to 12 weeks old animals, which did not reveal apparent signs of pancreatic β cell failure. However, it will be important to test, how pancreatic β cells lacking p38δ behave in the settings of type-2 diabetes. We will therefore cross the knockout mice with genetic models of obesity-related insulin resistance and also challenge them with a high-fat diet.

In conclusion, in our study, we identified for the first time an in vivo function for the non-canonical p38 isoform p38δ. We showed that p38δ suppresses insulin secretion from pancreatic β cells and therefore glucose tolerance. Increased insulin secretion from pancreatic β cells in the absence of p38δ was most probably caused by increased vesicle fission from the TGN membrane and was mediated by overactivation of PKD, since pharmacological blockage of PKD restored insulin secretion from p38δ-deficient islets to wild type levels.

Given the fact that p38δ has a specific expression pattern and its deletion in mice does not lead to any obvious detrimental health consequences, specific inhibition of p38δ might represent an attractive strategy to treat diabetic patients. However, additional experiments unraveling the exact function of this kinase in the models of type-2 diabetes need to be conducted.

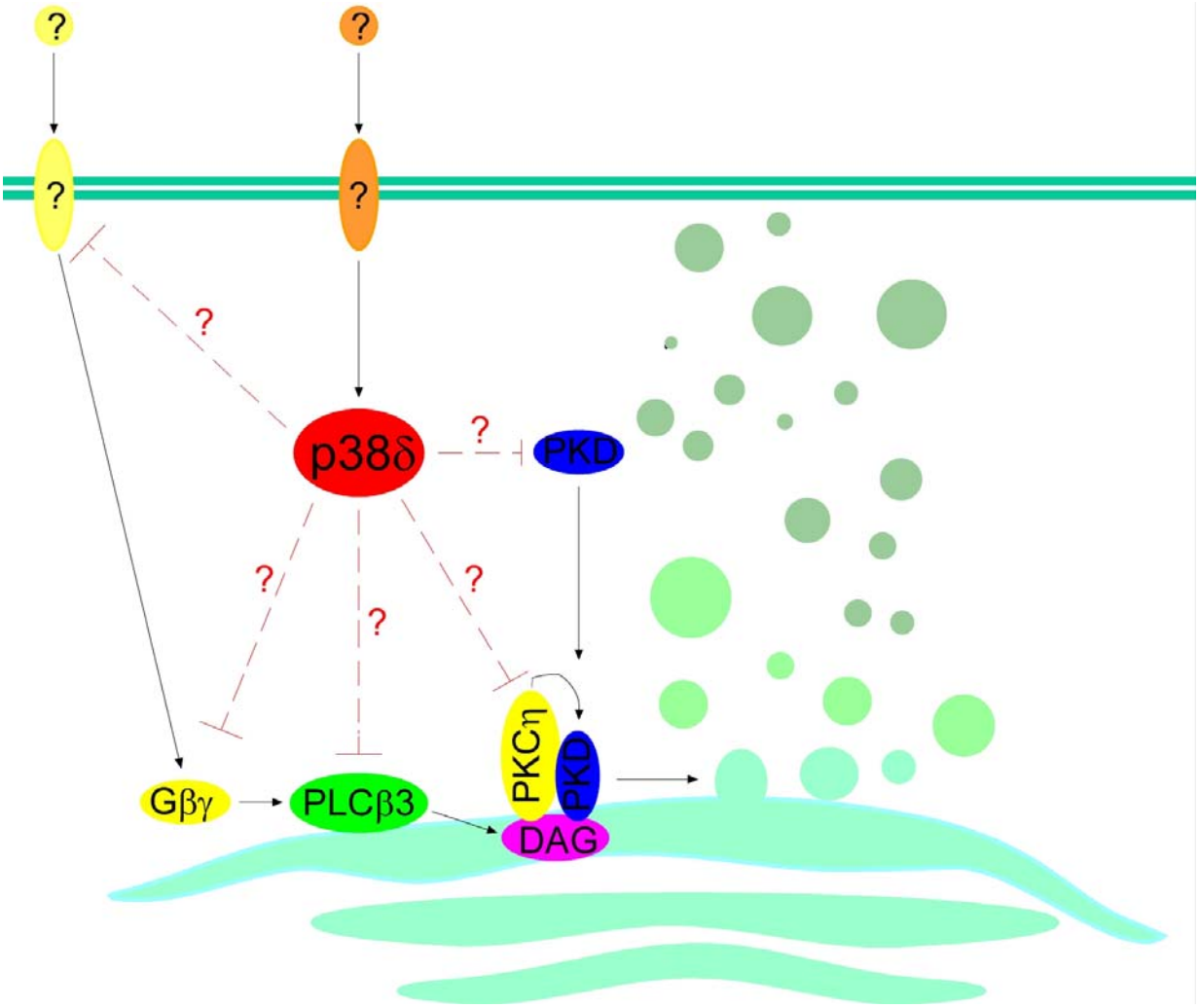

Fig. 21 Regulation of TGN membrane fission and possible targets of p38δ. Upon stimulation of cells, most probably mediated by a G protein coupled receptor (GPCR), GTP-binding protein βγ subunit translocates to the membrane of the TGN and activates phosphatidyl-inosytol phopholipase C β3 (PI-PLCβ3) resulting in generation of second messenger molecule, diacylglycerol (DAG) and subsequent activation of protein kinase C η (PKC η) and PKD. PKD mediates TGN membrane fission through not yet fully identified mechanisms. One possible target might be phosphatidylinositol 4-kinase IIIbeta (PI4KIIIbeta)[234]. Possible targets of p38δ are indicated with dashed lines. As indicated in the text, we favour a model, in which p38δ directly suppresses the activity of PKD.

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Curriculum vitae

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Publications

Original papers:

1. Ricci R*, **Sumara G***, Sumara I, Rozenberg I, Kurrer M, Akhmedov A, Hersberger M, Eriksson U, Eberli FR, Becher B, Borén J, Chen M, Cybulsky MI, Moore KJ, Freeman MW, Wagner EF, Matter CM and Lüscher TF.
Requirement of JNK2 for scavenger receptor A-mediated foam cell formation in atherogenesis. Science vol. 306 (1558-1561) Nov 2004 *equal contribution

2. Ricci R, Eriksson U, Oudit GY, Eferl R, Akhmedov A, Sumara I, **Sumara G**, Kassiri Z, David JP, Bakiri L, Sasse B, Idarraga MH, Rath M, Kurz D, Theussl HC, Perriard JC, Backx P, Penninger JM and Wagner EF.
Distinct functions of junD in cardiac hypertrophy and heart failure. Genes Dev. vol. 19 (208-213) Jan 2005

3. Stepniak E, Ricci R, Eferl R, **Sumara G**, Sumara I, Rath M, Hui L and Wagner EF. c-Jun/AP-1 controls liver regeneration by repressing p53/p21 and p38 MAPK activity. Genes Dev. vol. 20 (2306-2314) Aug 2006

4. Sumara I, Quadroni M, Frei C, Olma M, **Sumara G**, Ricci R and Peter M.
A Cul3-based E3 ligase removes Aurora B from mitotic chromosomes, regulating mitotic progression and completion of cytokinesis in human cells. Dev Cell. vol. 12 (887-900) Jun 2007

5. Falk S, Wurdak H, Ittner LM, Ille F, **Sumara G**, Schmid MT, Draganova K, Lang KS, Paratore C, Leveen P, Suter U, Karlsson S, Born W, Ricci R, Götz M and Sommer L.
Brain area-specific effect of TGFβ signaling on WNT-dependent neural stem cell expansion. Cell Stem Cell *in press*

6. **Sumara G**, Collins S, Sumara I, Formentini I, Musialek R, Remrachaya R, Caille D, Jiang H, Platt KA, Meda P, Rorsman P and Ricci R.
The MAPK p38δ is a negative regulator of insulin granule traffic in pancreatic β cells. *Manuscript in preparation*

7. Fuchs S, **Sumara G**, Büchmann-Moller S, Civenni G, Wurdak H, Suter U, Ricci R, Brakebusch C, Sommer L.
Cdc42 and Rac1 are essential for neural crest progenitor cell proliferation. *Manuscript in preparation*

Reviews:

8. **Sumara G**, Belwal M and Ricci R.
"Jnking" atherosclerosis. Review in Cell Mol Life Sci. vol. 62 (2487-2494) Nov 2005

Book chapters:

9. **Sumara G** and Balas M.
Fraktale (Fractals) Koktajl Matematyczny (Mathematical mix) book chapter in polish, 1999 (9-13)

10. **Sumara G** and Balas M.
Dualnosc (Duality) Koktajl Matematyczny (Mathematical mix) book chapter in polish, 1999 (15-20)

Invited presentations

1. Mitochondrial cardiomyopathy in mice overexpressing fra-1 and lacking junD. Poster presentation. 25th Annual Congress of the European Society of Cardiology. Vienna, Austria, Aug. 2003

2. The role of JNK signaling in arteriosclerosis: A possible therapeutic benefit in vascular disease? Poster presentation. The Suisse Cardiovascular research and training network annual meeting. Bern, Switzerland, Dec. 2003

3. JNK2 is required for scavenger receptor A-mediated foam cell formation and atherogenesis. Oral presentation. 2nd Annual Retreat of the Zürich PhD Program in Molecular Life Sciences. Lenk, Switzerland, Oct. 2005

4. Distinct functions of the p38delta MAPK isoform in acute and chronic inflammation and related diseases. 6th D-BIOL Symposium ETH Zürich. Davos, Switzerland, May 2006

5. p38δ – novel key regulator of innate immunity and glucose homeostasis. Poster presentation . 3rd Annual Retreat of the Zürich PhD Program in Molecular Life Sciences. Chandolin, Switzerland, Oct. 2006

Honours and Awards

1. The Roche Research Foundation PhD Fellowship, 2004

2. University scholarship awarded to 30% top students at the second, third, fourth and fifth year of study